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Department of Surgery

Germinal centre autoantibody responses following heart transplantation

Muhammad Saeed Qureshi

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Darwin College

University of Cambridge

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Declaration

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text. It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my thesis has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. It does not exceed the prescribed word limit for the relevant Degree Committee.

Abstract

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The development of humoral autoimmunity following organ transplantation is increasingly recognised, but of uncertain significance. I address the key question whether autoimmunity contributes independently to chronic graft rejection.

In a MHC class II-mismatched murine model, I show that antinuclear autoantibody responses were initiated upon graft-versus-host allorecognition of recipient B cells by ***donor*** CD4 T cells transferred within the heart allograft. Rejected allografts displayed features of chronic humoral vascular rejection, with rejection mediated by long-lasting germinal centre (GC) responses, whose maintenance was dependent upon additional, cognate help from ***recipient*** T follicular helper (T_{FH}) cells, and which diversified to encompass responses against vimentin autoantigen. Heart grafts transplanted into stable donor/ recipient mixed haematopoietic chimeras also provoked GC autoimmunity and were rejected with similar tempo, indicating that autoantibody can mediate graft damage despite host tolerance to alloantigen. An autonomous effector role for autoantibody was further suggested by the demonstration that F1 (BALB/c x C57BL/6) recipients reject parental BALB/c heart allografts, with rejection again dependent upon GC autoimmunity and help from recipient T_{FH} cells.

Thus GC autoantibody responses contribute to graft rejection independent of host adaptive alloimmunity, through a mechanism by which donor CD4 T cells initiate an auto reactive GC reaction that is then maintained and propagated by host T_{FH} cells. The demonstration that one set of CD4 T cells triggers autoimmunity, but that a second subset of T_{FH} cell is responsible for maintaining the response as a germinal centre reaction, has wider implications for our understanding of the pathogenesis of autoimmune disease.

Contents

Dedication

I dedicate this dissertation: to my father (Naseer Ud Din Qureshi), my mother (Asiya Baigum), my wife (Mehr Un Nisa), my son (Ebrahim) and my daughters (Maryam, Abeer and Minsa), for all their support and patience during the time I have devoted to this work.

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Abbreviations

7-AAD	7 – Aminoactinomycin D
Ab	antibody
AEC	amino-9-ethyl-carbazole
Ag	antigen
ADCC	antibody dependent cell-mediated cytotoxicity
AlloAb	alloantibody
ANA	anti-nuclear antibody
APC	antigen presenting cell
APC	allophycocyanin
ASCs	antibody secreting cells
AUC	area under the curve
BCR	B cell receptor
BM	bone marrow
BMC	bone marrow cell
BSA	bovine serum albumin
AV	allograft vasculopathy
C	constant domain
CCL	CC-chemokine ligand
CCR	CC-chemokine receptor
CDC	complement dependent cytotoxicity
CFSE	carboxyfluorescein succinimidyl ester
CXCL	CXC-chemokine ligand
CXCR	CXC-chemokine receptor
D	diversity segment
DC	dendritic cell
DMF	dimethylformamide
ELISA	enzyme-linked immunosorbent assay

ELISPOT	enzyme-linked immunosorbent spot
EVG	elastin van Gieson
Fc	constant-region fragment
FCS	foetal Calf Serum
FDC	follicular dendritic cell
FITC	flourexcein isothiocynanate
GC	germinal centre
GVH	Graft versus host
HLA	human leukocyte antigen
ICOS	inducible T cell costimulator
IEL	internal elastic lamina
Ig	immunoglobuloin
IP	intraperitoneal
IV	intrcavenous
IVC	inferior vena cava
J	joining segment
LLPC	long-lived plasma cell
mAb	monoclonal antibody
2ME	2 mercaptoethanol
MHC	major Histocompatibility Complex
MST	median survival time
PBS	phosphate Buffered Saline
PE	phyocoerythrin
RAG	recombination activating gene
rpm	revolutions per minute
RPMI	Roswell Park memorial institute medium
RT	room temperature
SAP	SLAM associated protein
SLAM	signalling lymphocytic activation molecule

T_{CM}	central memory T cells
TE_M	effector memory T cells
TCR	T cell receptor
$TCR^{-/-}$	T cell receptor knock out
T_{FH}	T follicular helper
Tg	transgenic
TLR	toll-like receptor
TMB	tetramethylbenzidene
T regs	regulatory T cells
WT	wild type
SHM	somatic hypermutation
V	variable domain

Presentations

Regional

Germinal centre responses drive epitope-diversification in an autoantibody mediated model of heart allograft vasculopathy at Research Day for University of Cambridge PhD students, Poster, 26/06/2013 (**first author**)

Host CD4 T cells are essential for diversification ofdonor induced autoimmunity Poster, Presented, Research Day for University of Cambridge PhD students, 22/06/2012, prize session **(first author)**

National

Contribution of donor and host CD4 T cell populations to initiation and diversification of transplant-associated autoantibody responses, oral presentation (prize session) at the British Society of Transplantation, Glasgow, 2014 **(first author)**

Contribution of donor and host CD4 T cell populations to initiation and diversification of transplant-associated autoantibody responses, oral presentation (prize session) at SARS annual meeting Cambridge, 2014 **(first author)**

Germinal centre responses drive epitope diversification in an autoantibody-mediated model of heart allograft vasculopathy, oral presentation at the British Society of Transplantation, Bournemouth, 2013. **(first author)**

The role of host CD4 T cells in germinal centre mediated autoantibody production in GVH responses in Medawar Medal Session, Oral presentation (prize session), the British Transplantation Society Annual Congress, Glasgow, Feb 2012 (first author).

International

Germinal centre autoimmunity mediates progression of allograft vasculopathy, with essential help being provided by T follicular cells. ESOT 2015 at Brussels oral presentation **(first author).**

Contribution of donor and host CD4 T cell populations to initiation and diversification of transplant-associated autoantibody responses. Poster, Presented at WTC 2014 at San Francisco **(first author but presented by my colleague)**.

Germinal centre responses drive epitope diversification in an autoantibody-mediated model of heart allograft vasculopathy, oral presentation at European Society of Organ Transplant, Vienna, 2013 **(first author).**

Germinal centre responses drive epitope diversification in an autoantibody-mediated model of heart allograft vasculopathy, oral presentation at American Transplant Congress, Seattle 2013. **(first author but presented by my colleague)**

T-Cell Help Is Critical in Determining Mode of Alloantibody-Mediated Allograft Rejection, oral presentation at American Transplant Congress, Seattle 2013 **(first author but presented by my colleague).**

The Role of Donor and Recipient CD4 T Cells in the Initiation and Maintenance of Autoantibody after Heart Transplantation- oral presentation at International Transplantation Society Congress Berlin, 2012 **(first author).**

Awards and prizes

Regional Awards

2012 **Second prize** for **First Year PhD students' poster presentations** at School of Clinical Medicine, University of Cambridge Jun 2012

National Awards

2012 **British Heart Foundation Clinical Research Training Fellowship Award** (£232,700)

2012 The **Best Science abstract** for highest scoring for science abstract by **the British Transplantation Society (BTS) 2012, at Glasgow** (Prize money £500)

2014 Finalist for '**Medawar Medal' at the British Transplantation Society (BTS-2014, Glasgow)** for oral presentation of basic science research

2014 Finalist for the highest surgical award 'PATEY PRIZE' at **Society of Academic and Research Surgery annual meeting (SARS)** for oral presentation at SARS, 2014, Cambridge

International

2014 **"Poster of Distinction**" at world transplants Congress, San Francisco, for my Basic Science Research, 2014, San Francisco

Publications

M. Saeed Qureshi, Jawaher Alsughayyir, Manu Chhabra, Jason M Ali, Martin J. Goddard, Chris Devine, Thomas M. Conlon, Michelle A. Linterman, Reza Motallebzadeh, Gavin J. Pettigrew. **Germinal centre humoral autoimmunity independently mediates progression of allograft vasculopathy.** Journal of autoimmunity 98(2019) 44-58 (Qureshi et al., 2018)

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1. Introduction

1.1. Challenges in solid organ transplantation

Solid organ transplantation is one of the most remarkable and dramatic therapeutic achievements in medicine in the last 6 decades. It offers patients with end stage organ failure a cost effective treatment that improves quality of life and life expectancy.

It was not possible till 1954, when the first successful kidney transplant was performed by Dr. Joseph Murray (Harrison et al., 1956), between a patient and his identical twin brother. With the subsequent advances in organ procurement, surgical techniques, immunosuppression and organ preservation, transplant outcomes have continually improved, leading to an increasing demand for organs. Several approaches have been adopted to try and meet this demand, but strategies that successfully improve long-term transplant outcomes would undoubtedly make a huge difference. Chronic rejection remains the leading cause of transplant loss, and as many as a quarter of adult UK patients awaiting kidney transplantation have already received at least one kidney. However chronic rejection remains a poorly understood process and the underlying mechanisms that lead to graft loss have not yet been clarified. Immune recognition of mismatched donor antigens is thought to be a critical aspect of chronic rejection, but it is notable that it is now very unusual, with modern immunosuppression, for a transplant to fail from acute rejection, and yet the incidence of chronic rejection has not changed.

Consequently, patients generally remain on immunosuppression for the life of the transplant. This approach is not ideal, because current nonspecific immunosuppression is associated with increasing opportunistic infections and neoplasm in the recipients. Hence, in the current scenario, when more marginal organs are being used to compensate for organ shortage, it has become all the more important to improve our understanding of the pathogenesis and molecular pathways that govern chronic rejection. This will allow us to develop more tailored and more effective therapeutic strategies, which will finally improve long term outcomes for transplant patients.

1.2. Immunology in transplantation

Adaptive immunity against donor antigens is a major obstacle to organ transplantation. It became apparent from initial work of Medawar and his colleagues that lymphocytes are the principal mediators of rejection (Gowans et al., 1962; Medawar, 1944). Later, Snell and Gorer's ground-breaking work in tissue transplantation led to the discovery of the major histocompatibility complex (MHC)(Snell, 1948). MHC molecules on the donor are the principal target of the alloimmune response in transplantation.

1.2.1. Development of T and B lymphocytes

T and B lymphocytes are involved in antigen specific immune responses. They are the only cells in the organisms which are capable to recognise and respond specifically to each antigenic epitope. B cells differentiate into plasma cells or plasmablasts and produce antibodies, thus are involved with humoral immunity and T cells are central to cellular immunity. Below I will discuss their central development and differentiation into functional cells.

1.2.1.1. Development of T cell in thymus

T cells are derived from haematopoietic stem cells that are initially found in the fetal liver and then in the bone marrow. Only a small subset of common lymphoid progenitors migrate to the thymus and differentiate into early thymic progenitors, also known as thymocytes. Thymocytes undergo a series of maturation steps within the thymus that can be identified based on the expression of different cell surface markers. The majority of the cells in the thymus give rise to αβ receptor T cells, however a small percentage does bear γδ T cells. During this process of development, thymocytes interact with the stromal cells and undergo process of maturation as described below in different anatomical locations of the thymus. Thymus is made of subcapsular region, cortex, cortico-medullary junction and medulla.

The stem cell that has committed to become T cells, thymocytes lack expression of the co-receptors CD4 and CD8 molecules and are termed a double negative (DN) cells (Yang et al., 2010). These DN can be further subdivided by the expression of CD44 and CD25. Cells that lack expression of CD44 but possess CD25 undergo β-selection process (DN3). It is important to note here that multiple gene segments for individual chains (α, β, γ and δ) dispersed in the genomic DNA must bind and transcribe to produce a functional TCR. All the chains

undergo this process of recombination independent of each other and begins with recombination of genes for the β chain (Abbey and O'Neill, 2008). TCR genes are assembled through V(D)J recombination. This is a site specific recombination process directed by the lymphoid-specific recombinase (RAG1 and RAG2) and DNA repair proteins. RAG proteins create double-strand breaks at recombination signal sequences (RSSs) that flank TCR variable (V), diversity (D) and joining (J) gene segments, and these breaks are subsequently resolved by nonhomologous end joining. During this process, β chain loci recombination competes with γδ recombination. In this competition normally β chain succeeds and stops recombination of γδ chain. In cases where γδ chain recombination is productive, γδ T cells exit from thymus as γδ T cells at this point, constituting approximately 5% of the total T cells (Carding and Egan, 2002; Vantourout and Hayday, 2013). If Beta chain recombination is productive then it pairs with the surrogate alpha chain called pre-Tα to produce a pre-TCR which forms a complex with CD3 molecules. This complex leads to the survival, proliferation, arrest in further β chain loci rearrangement and upregulation and expression of CD4 and CD8 molecules thus making them double positive (DP) cells (Fehling et al., 1995; Rothenberg et al., 2008; Saint-Ruf et al., 1994). β-selection process is considered as a check point to identify whether this β chain is functional or not. If pre-TCR is functional then it forms dimers and initiates signaling which results in turning on α chain recombination and turning off βγδ recombination. Cells that do not undergo beta-selection die by apoptosis. Double positive cells (DP) re-express RAG genes which initiate TCRα recombination to produce αβ-TCR (Klein et al., 2009).

Double positive cells then undergo positive selection in thymic cortex in which thymic epithelial cells present self-restricted peptides in the context of class I and class II MHC molecules (Anderson and Takahama, 2012). Only a small fraction of these DP lymphocytes which bind to the self-peptides with intermediate affinity survive while the others which are incapable of binding to MHC molecules undergo apoptosis. As a result of this selection process, DP cells that recognise MHC class I differentiate into $CD4^-$ $CD8^+$ and DP cells that recognise MHC class II differentiate into $CD4^+CD8^-$ cells. Subsequently these single positive (SP) cells entre into thymic medulla where they undergo negative selection process in which these SP cells are exposed to a diverse set of self-antigens presented by medullary thymic epithelial cells and antigen presenting cells (APCs), such as dendritic cells and macrophages.

Single positive T cells that interact with high affinity or avidity to self-antigens are eliminated by apoptosis thus ensuring central tolerance. Following selection, down regulation of either co-receptor produces either naïve CD4 or CD8 single positive cells. Naïve T cells leave the thymus and migrate continuously to the secondary lymphoid organs to be primed by antigen and differentiate into effector T cells.

T cell receptor complex is composed of αβ or γδ variable chains which are associated with invariant accessory proteins (CD3γε, CD3δε and CD247 ζζ chains) (Wucherpfennig et al., 2010). TCR α and β chains are very polymorphic, which enable T cells to recognise a wider repertoire of antigenic peptides. Each chain is made of a variable (V) and a constant domain (C) with a joining segment (J) that lies between them. The β chain also has an additional diversity segment (D). Each V domain has three hypervariable regions known as CDR1, CDR2 and CDR3 (complimentarity-determining regions). These hyervariable regions undergo recombinations and result in the generations of different TCRs specific for a particular antigen. CDR3α and beta regions bind to the central region of the antigenic peptide presented and is the most diverse part of the TCR and considered the main determinant of specificity in antigen recognition. CD3 chains and the CD247 ζζ chains that associate with TCR possess tyrosine based activation motif (ITAMS) in their intracytoplasmic tails which are phosphorylated to initiate T cell activation (Baniyash, 2004).

1.2.1.2. Development of B cell

Early B cell development and commitment to B cell lineage initiates in the foetal liver prenatally, thereafter it continues within the bone marrow throughout the life of animal. B cells play a pivotal role in adaptive immunity and are responsible for mediating the production of antigen specific immunoglobulins i.e. antibodies which help fight against invasive pathogens. The function of B cells was discovered in 1960s (Cooper et al., 1965) by Max Cooper et al after a series of experiments in birds, mammals and immunodeficient patients by showing that antibody production was completely abrogated in irradiated chickens after surgical removal of the Bursa of Fabricius. Bursa of fabricius is the primary site for B cell development in birds and from which the notation ‘B’ cell was derived.

Haematopoietic stem cells (HSC) which are committed to become B cells differentiate into early lymphoid progenitor (ELP) which further differentiate into common lymphoid

progenitor (CLP). CLP on one end lead to development of DC and NK cells and on the other, differentiate into CLP-2 which is responsible for B cell lineage. This is considered the first stage of the immature B cells. A prerequisite for the development of B cells in the bone marrow is the suppression of protein Notch-1 (N1) signaling, which is necessary for T cell development (Cruickshank and Ulgiati, 2010). From here their development into B cells occur in several stages. Functional B cell receptor development is dependent on productive and sequential rearrangement of a heavy and light chain gene directed by RAG proteins. Immunoglobulins consist of two identical heavy and light chains, which are joined by di-sulphide bonds. CLP and early B cells undergo Ig heavy chain recombination, first commencing with D-J recombination on both chromosomes (paternal and maternal). Non-productive rearrangement will lead to apoptosis of early B cells at this stage while productive rearrangement on both chromosomes leads to V-DJ recombination. V-DJ recombination takes place on the first chromosome (maternal/paternal). If this recombination fails then V-DJ recombination takes place on the second chromosome. As a result of successful V-DJ recombination, 50% of the cells are signaled to survive and to become large pre-B cell. Due to non-productive V-DJ recombination, ½ of the cells undergo apoptosis. The recombined heavy chain then associates with the surrogate light chains to check that heavy chain can form the di-sulphide bond with this surrogate light chain which is structurally similar to light chain. At this stage, pre B cell receptor (pre-BCR) is expressed on the cell surface. Signalling through the pre-BCR drives intense proliferation and as a result, significantly increases their number and guarantees that they successfully carry out the heavy chain recombination. Furthermore, signalling through pre-BCR directs activation of the gene recombination for the light chain(von Boehmer and Melchers, 2010). These pro-B cells thus differentiate into the small pre-B cell stage (Herzog et al., 2009). Small pre-B cell then undergo V-J rearrangement of the Ig light chain genes starting with kappa chain gene (κ chain) first and if the recombination is productive, the immature B cell expresses the μ chain and the κ chain which results in functional IgM. If the κ chain gene rearrangement was not productive then lambda chain genes (λ) undergo V-J rearrangement. As a result of successful rearrangement on λ chain gene, IgM+ immature B cell expresses μ chain and the λ chain. The odds of failing a light chain gene rearrangement is very low. The ones who have survived the heavy chain rearrangement, will survive the light chain rearrangement as well.

The fate of immature B cells is determined by the strength of B cell signalling (Yurasov et al., 2005). In the absence of a signal that is stronger than that provided by tonic BCR signaling, the cells exit the BM as immature transitional B cell towards secondary lymphoid organs where they undergo final differentiation and maturation through distinct transitional B cell stages termed as T1 and T2 before differentiating into long lived mature follicular (FO) or marginal zone (MZ) B cells (discussed in detail below in section 1.2.3). Thus, B cells undergo antigen dependent and independent phases of differentiation and maturation and each phase tightly regulated by signalling events. Apart from T1 and T2 B cells, there is another subset of B cells termed as T3 B cells which represent a subset of anergic B cells which have been selected away from the development pathway. With regards mature B cells, in addition to FO and MZ mature B cells, there is another population of mature B cells called as B1 cells which is found in the spleen, intestine, peritoneal cavity and pleural cavity but less commonly in the blood. B1 cells have a distinct haematopoietic origin in the fetal liver and appear to play a role during an infection or vaccination (Cunningham et al., 2014).

Within the bone marrow, immature B cells undergo central tolerance to prevent autoimmunity by clonal deletion/receptor editing or clonal anergy. B cell receptor that bind with multivalent self-antigens with strong affinity result in clonal deletion or receptor editing while immature B cells that recognise monovalent self-antigen usually soluble proteins in the blood vessel become non-responsive or anergised and are characterized by IgD expression with little to no IgM.

Receptor editing is a major mechanism of central tolerance in B cells in which a productively rearranged immunoglobulin light chain or heavy chain variable region gene undergo replacement with another one , either on the same chromosome, on the homologous chromosome or through an isotype switch (κ light chain to λ light chain) (Luning Prak et al., 2011). During this process if a developing B cell expresses a heavy/light chain combination that recognise a self -antigen with sufficient affinity , it can be signaled to continue to express Ig gene recombination machinery including RAG1 and RAG2 genes. This then undergo further rearrangement that replace either the light chain or heavy chain variable regions so that a new BCR is produced that is no longer self-reactive. These immature B cells then migrate to the periphery as transitional B cell stages termed as T1 and T2 before differentiating into long lived mature follicular (FO) or marginal zone (MZ) B cells. The role of

receptor editing in the secondary lymphoid organ is controversial. The receptor editing in the periphery is also termed as receptor revision. The evidence for this is the expression of RAG gene in the spleen and lymph nodes especially after an antigenic challenge (Yu et al., 1999). Furthermore, in vitro experiments in mice have suggested that RAG genes can be upregulated in B cells by stimulating them with LPS and IL-4 (Hikida et al., 1996). Similarly, in autoimmune mice RAG genes were reinduced in antigen activated early memory or preplasma B cells which helps to prevent autoimmunity in the periphery (Wang and Diamond, 2008). However, in another study peripheral receptor editing resulted in the development of an autoantibody (Brard et al., 1999).

1.2.2. Allorecognition in transplantation

Allorecognition refers to the recognition of donor antigens by the recipient T cells. The transplant antigens can be major histocompatibility (MHC) antigens or minor histocompatibility antigens (MiHA) which can be recognized by CD4 or CD8 T cells by different pathways of allorecognition.

There are three classes of MHC genes (HLA in humans and H-2 in mice); but only class I and class II are involved in antigen presentation. *MHC class I* consists of two polypeptide chains, α and β2-microglobulin. Only the α chain is polymorphic and encoded by a HLA gene, while the β2-microglobulin subunit is not polymorphic and encoded by the Beta-2 microglobulin gene. The class I α chain is composed of three extracellular domains (α1, α2, and α3), a transmembrane segment, and a short cytoplasmic tail (Bjorkman et al., 1987). The α1 and α2 domains fold together to form a deep groove that constitutes the peptide binding site. The bound peptide and associated regions of the α1 and α2 domains interact physically with the T-cell receptor (Wu et al., 2002). The amino acid sequence of α3 domain interacts with β2-microglobulin and also serves as binding site for the CD8 co-receptor, which facilitates the interaction of T cells with MHC class I molecules. The peptides which associate with MHC class I molecules have preferential anchor residues, which are important for binding to specific MHC class I alleles (Gavioli et al., 1995; Haurum et al., 1995). This allows binding by a wide repertoire of peptides. The cytoplasmic tail of MHC class I molecule plays a critical role in intracellular trafficking and in dendritic cell-mediated antigen presentation and cytotoxic T lymphocyte (CTL) activation.

MHC Class II molecules are composed of an α chain and a β chain. The α-chain and β-chain of MHC-II is a membrane bound glycoprotein that contains external domains, a transmembrane segment and a cytoplasmic tail. The α chain and β chain are made up of two domains (α1 and α2) and (β1 and β2) respectively. The α1 and β1 regions of the chains associate to form a membrane-distal peptide-binding domain, while the α2 and β2 regions, the remaining extracellular parts of the chains, form a membrane-proximal immunoglobulin-like domain. The antigen binding cleft, composed of two alph-helicles above a beta pleated sheet, specifically binds short peptides, about 15-24 residue long. The amino acid sequence around the binding site, which specifies the antigen binding properties, is the most variable site of the MHC molecule. All nucleated cells express MHC class I, whereas MHC class II proteins are expressed mostly on dendritic cells (DCs), B cells, and can be induced on endothelial cells. Classically, class I molecules present peptides of endogenous proteins to CD8 T cells and class II molecules to CD4 T cells.

Minor histocompatibility antigens are normal proteins that are encoded by many chromosomes. Even when the transplant donor and recipient are identical at their MHC loci, differences in amino acid sequence on these minor histocompatibility antigens can lead to rejection. As a consequence of mismatches at minor HC antigens, immunosuppression is still required in recipients of organs from HLA matched, non-identical twin siblings. The prototypic example of a minor antigen is the H-Y antigen, which is derived from group of proteins encoded by the Y chromosome. Allo-responses to this minor antigen may explain reduced long term survival of allografts in female recipients of male grafts (Gratwohl et al., 2008). Other examples of minor histocompatibility antigens are MHC I-related chain antigens (MICA), ABO blood groups glycoproteins, and autoantigens. The MHC I-related chain antigens are surface glycoproteins and encoded by MICA gene. These are expressed on the surface of endothelial cells and are thought to be involved in innate immunity. These proteins are ligands for the activating receptor NKG2D on NK cells, CD8 T cells, and gamma/delta T cells. Exposure of allogenic MICA proteins in transplantation can elicit antibody formation in recipients (Zou et al., 2006). Pre-sensitization of kidney-transplant recipients against MICA antigens is associated with an increased frequency of graft loss and might contribute to allograft loss among recipients who are well matched for HLA (Zou et al., 2007). The ABO blood group glycoproteins are other MiHA which are expressed on the

surface of endothelial and red blood cells. Finally immune responses to autoantigens have been reported to be associated with allograft damage (Dave and Bayless, 2014; Win and Pettigrew, 2010; Zhang and Reed, 2016).

T cells recognise alloantigens by two pathways; the direct and indirect pathway of allorecognition (Rogers and Lechler, 2001). In the direct pathway, the intact donor MHC class I and class II are presented by donor antigen presenting cells (APCs), and are recognised by recipient CD8 or CD4 T cells respectively. In the indirect pathway, recipient CD4 T cells recognise endogenously processed allopeptide fragments presented in the context of host MHC class II by recipient APCs. In comparison, the precursor frequency of T cells that can respond via the indirect pathway of allorecognition is similar to self-restricted responses against conventional protein antigens (such as viral proteins), and approximately100 fold less than that observed in direct allorecognition (Liu et al., 1993). The direct pathway CD4 T cell responses are thought to be the principal contributor to early rejection episodes due to the high precursor frequency of alloreactive T cells in the periphery (Bach and Hirschhorn, 1964; Ichikawa et al., 1987; Rosenberg et al., 1987; Rosenberg et al., 1991). Consequently, the indirect pathway CD4 T cell responses are thought to be principally involved in chronic rejection (Conlon et al., 2012; Haynes et al., 2012).

1.2.3. Generation of humoral immune response and germinal centres

Humoral immune response is mediated by antibodies produced by B cells against the invading extracellular microorganisms and prevent the spread of intracellular infections. In transplantation, antibodies have been thought to be responsible for chronic rejection of organs (discussed below in section 1.3). Antibodies are generated as result of B cell activation following interaction with an antigen. The development of antibodies is remarkably complex, with a number of different B cell subpopulations at different anatomical locations (B1 (Herzenberg, 2000), marginal zone (Martin and Kearney, 2002), extrafollicular (MacLennan et al., 2003) and germinal centre (GC) (Allen et al., 2007a) contributing to varying degrees to the overall antibody response.

Following maturation of the B cells in the bone marrow and spleen (discussed in section 1.2.1.2), immunocompetent B cells remain in peripheral tissues until they are activated by

an antigen. B cell activation requires two distinct signals; the first activation signal occurs upon antigen binding to B cell receptors. Upon binding to the BCR, the antigen is internalized by receptor-mediated endocytosis, digested, and complexed with MHC II molecules on the B cell surface. The second activation signal occurs via either a thymus-dependent (T-dependent) or a thymus-independent (T independent) mechanism. In a T-independent immune response B cells can respond directly to the antigen like some bacterial cell wall components (e.g., lipopolysaccharide) or antigens containing highly repetitious molecules (e.g., bacterial flagellin). In a T-dependent immune response, the B cells need help from T cells in order to respond.

Marginal zone (MZ) B cell response to antigen is thought to be T- independent and is a crucial component of the early response to blood borne antigens. This B-cell subset helps to bridge the temporal gap between the innate immune response and adaptive immune responses. They are charctersied by IgM^{hi}, IgD^{low}, $CD21^{hi}$ and $CD23^{low}$ surface markers and mostly are non-recirculating (Berland and Wortis, 2002; Martin and Kearney, 2000). Anatomically, marginal zone B cells are separated from mature follicular B cells by the marginal sinus. In addition to being anatomically distinct from other the other cell types, MZ B-cells exhibit a high antigen-presenting capacity (Oliver et al., 1999; Tanguay et al., 1999), and preferentially secrete complement-fixing, multi-reactive immunoglobulin (Hardy and Hayakawa, 2001). Humoral response generated from marginal zone B cell arm is thought to play an important role in

For T dependent humoral responses, there are two main mechanisms by which B cells form antibodies; extrafollicular pathway (EF) or germinal centres (GCs), depending on the type of help B cells receives from CD4 T cells (Garside et al., 1998). Upon stimulation of BCR, B cells secrete macrophage inflammatory protein (MIP-1α) and dendritic cell chemokine-1(ABCD-1), which are T cell chemoattractants (Cyster, 1999). This promotes T-B cell interaction at T-B cell border of lymphoid organs. This involves a cognate, physical linkage between the MHC class II-peptide complex of the B cell and T cell receptor of the alloantigen. At T-B border, B cells which have sustained interactions with CD4 T cells through signalling-lymphocyte-activation molecule (SLAM) receptors migrate to B cell follicles to develop into germinal centres (Qi et al., 2008) while others migrate to the medullary cords in the lymph nodes or the bridging channels in the spleen to form extrafollicular foci (EF pathway). Extrafollicular plasma cells secrete class-switched antibody for around three days, after which the majority undergo apoptosis (Smith et

al., 1996). But a small fraction can live for longer periods in the secondary lymphoid tissues (Sze et al., 2000). This initial burst of antibodies with modest affinity for antigen plays an important role in the early control of infection (Luther et al., 1997).

Long lasting antibodies are the result of germinal centres (GCs) which are characterised by a light zone and a dark zone. The germinal centre response begins in the dark zone where the B cells rapidly proliferate and are called centroblasts. These B cells undergo somatic hypermutation during which random mutations are generated in the variable domains of the BCR by the enzyme activation-induced cytidine deaminase (AID) (Berek et al., 1991; Muramatsu et al., 2000). B cells then enter the light zone and compete with each other for antigen. If the mutation resulted in a BCR with an improved affinity to the antigen, the B cell clone can out-compete other clones and survive. However, B cells which lack this high affinity recirculate between the dark and light zone to achieve high affinity BCR against the antigen by undergoing rounds of somatic hypermutations or die by apoptosis. Within the light zone, the B cells (centrocytes) which survive move about the FDCs network and pick up antigens, receiving pro-survival BCR signals (Allen et al., 2007a) thus internalising, processing and presenting the antigen as peptide complex in the context of MHC class II (MacLennan, 1994). In doing so, the high affinity B cells may be able to pick more antigen than low affinity B cells before internalisation. Then these GC B cells compete with other GC B cells and dead B cell blebs for follicular helper CD4 T cell (T_{FH} cell) help in the light zone. It is now widely accepted that GC are dependent on special subset of CD4 T cells for their development called follicular helper CD4 T cells (T_{FH} cell) (Yu and Vinuesa, 2010). The T_{FH} cell in GC is positioned in such a way that only one T_{FH} makes stable interaction with the B cell which bears the highest number of the antigen peptide complex on MHC class II molecule (Allen et al., 2007a; Allen et al., 2007b). The centrocyte that receives help from T_{FH} cell can either exit the GC as LLPC and memory B cells or return to the dark zone for further affinity maturation (Kelsoe, 1996; Victora et al., 2010). However, if centrocyte B cell does not receive help from T_{FH} cell at this stage, the response collapses (de Vinuesa et al., 2000; Qi, 2012).

The widely accepted view is that LLPCs are exclusively a consequence of a GCs response (Good-Jacobson et al., 2010; Han et al., 1995; MacLennan, 1994; Takahashi et al., 1998; Vinuesa et al., 2009). On immunostaining, these GCs have been characterised by PNA and GL 7 positivity, and it has been suggested that activated germinal centre B cells can be probed with peanut

(*Arachis hypogaea*) lectin (Rose et al., 1980), peanut agglutinin (PNA) (Coico et al., 1983; Muramatsu et al., 1999) or a rat monoclonal antibody (MAb), GL7 (Cervenak et al., 2001). PNA is a lectin with specificity for terminal galactosyl residues and it has been found that GC B cells have high levels of PNA compared to other lymphocytes. Similarly, GL7 was originally reported as a marker of activation for polyclonal T and B cells in mice (Laszlo et al., 1993). GL7 was also found to stain a subpopulation of T cells (Han et al., 1996) and a subpopulation of the large pre-B-cell stage during differentiation in the bone marrow (Murasawa et al., 2002). However, activated B cells express the GL7 epitope, but mature B cells do not; thus, GL7 serves as a marker for germinal centres in the immunized spleen (Han et al., 1996; Pasare and Medzhitov, 2005; Shapiro-Shelef et al., 2003) or lymph nodes. Functional comparison between $GL7^{hi}$ and $GL7^{lo}$ splenic B cell population in immunised mice showed significantly more specific and higher total antibody production as well as antigen presenting capacity in the $GL7^{hi}$ population (Cervenak et al., 2001). In addition to the LLPC, GCs generate memory B cells, which are another aspect of long lived humoral immunity. Importantly, within the germinal centre, B cells undergo somatic hypermutation (SHM) of immunoglobulin (Ig) variable (V) region genes, and the subsequent selection of B cells based on the affinity of the encoded B-cell receptor (BCR) for the eliciting antigen (Ag) results in affinity maturation (Berek et al., 1991; MacLennan, 1994).

The T_{FH} cell is critical for the initiation and maintenance of the GC response, but debate persists regarding their signature markers. Critical to their function is their ability to localise within the germinal centre follicle. This is achieved by upregulation of CXCR5; the receptor for B cell follicle-produced chemokine CXCL13 (Breitfeld et al., 2000; Schaerli et al., 2000; Vinuesa et al., 2005), and is probably expressed as a consequence of the T-B cell cognate interaction that occurs at the T-B cell border at the onset of the response (Garside et al., 1998). Within the follicle, the T_{FH} cell sustains the germinal centre response by providing essential survival signals to centrocytes (again through cognate interaction with the peptide-MHC II complex of the B cell (Vinuesa et al., 2009)). The number of T_{FH} cells is extremely limited and only high affinity helper T cells are selected to become T_{FH} cells (Fazilleau et al., 2009). Thus competition for the help they provide is postulated as one of the main mechanisms for driving affinity maturation, because only those B cells that bind target

antigen most strongly present enough peptide to receive effective help (Allen et al., 2007a; Allen et al., 2007b).

Aside from CXCR5, T_{FH} cells express a number of markers that distinguish them from other CD4 T cell subsets and which could in theory be targeted to achieve selective depletion. B cell lymphoma 6 (Bcl-6) has been described as the master transcription factor (Crotty et al., 2010; Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009), and is induced by interaction with the B cell. Germinal centres do not develop if the T cell population is deficient in Bcl-6 expression (Johnston et al., 2009; Yu et al., 2009). Equally critical is expression of signalling-lymphocyte-activation molecule associated protein (SAP, (Qi et al., 2008)). T cells that lack SAP expression do not form the same stable, initial interactions with B cells at the T-B border as can be visualised with SAP^{+ve} T cells (Qi et al., 2008), and do not migrate into the follicle. Without SAP signalling, the GC response is curtailed. IL-21 secretion also appears to be fundamental for follicular helper T cell function (Linterman et al., 2010; Nurieva et al., 2008; Schmitt et al., 2009; Vogelzang et al., 2008; Zotos et al., 2010) and in its absence the GC response is terminated rapidly with production of low affinity antibody. Finally both ICOS and PD-1 expression also characterise the T_{FH} cells subset (Good-Jacobson et al., 2010; King et al., 2008).

1.2.4. Immunological tolerance and transplantation

Immunological tolerance is basically a state of unresponsiveness in which the lymphocytes may remain alive but cannot mount an effective immune response against an invading antigen which is achieved by central and peripheral mechanisms. In central tolerance, lymphocytes with receptors specific for self-antigens are deleted/edited or become anergic during their development at an early stage (discussed in section 1.2.1). Furthermore some $CD4^{+ve}$ T cells receive signals in the thymus that select them to differentiate into natural T regulatory (nTRegs), which express the FoxP3 transcription factor and suppress the immune response by doth direct and indirect mechanisms (Ferrer et al., 2014; Louis et al., 2006; Nafady-Hego et al., 2010; van der Net et al., 2016). Escape of the self-reactive T cells and B cells into the periphery may result in autoimmunity. However, these are deleted by apoptosis or become anergic or are kept under control by natural regulatory T cells or induced T regulatory cells (iTRegs) (Curotto de Lafaille and Lafaille, 2009). Induced T regulatory cells

have similar effector function as nTRegs but they are produced in the periphery (Romano et al., 2019; Schmitt and Williams, 2013).

In transplantation, a small group of recipients display stable graft function in the absence of immunosuppression, while maintaining an intact immune response to infections. This phenomenon is called operational tolerance. Operationally tolerant recipients have displayed differences in gene expressions, peripheral blood cell populations and transcripts compared to immunosuppressed patients in chronic rejection and healthy volunteers (Sarwal, 2016). Unique genetic expression has been identified from tolerant kidney and liver recipients compared to those with chronic rejection (Brouard et al., 2007; Martinez-Llordella et al., 2007). This could potentially help identifying the potential candidates for reducing dependence on immunosuppression (Roedder et al., 2015).

Furthermore the concept of divided tolerance or split tolerance was described in late 1950s in which there was selective acceptance of syngeneic C57BL male skin graft by C57BL female who were immunised at birth with allogenic cells derived from A strain males but rejection of donor skin grafts from A strain males (Lustgraaf et al., 1960). This concept has been further studied by others in which one organ may be accepted by a recipient while grafts of other tissues from the same donor were rejected (Chan et al., 2008; Chung et al., 2005; Luo et al., 2007; Mathes et al., 2003; Qian et al., 1997). Although mechanisms leading to split tolerance are not well understood, but is applicable to the immunological relationship between mother and foetus. De Mastre et al (de Mestre et al., 2010) studied this relationship in pregnant mares in which they found that antibody responses to paternal MHC class I antigens were robust while anti-paternal cytotoxic T cell responses were diminished compared to those mounted by non-pregnant mares. Furthermore they demonstrated that a state of split tolerance exist in trophoblast tissue in endometrial cups which is maintained by higher number of interferon gamma positive lymphocytes and FOXP3 positive cells in endometrial cups compared to the maternal peripheral blood lymphocytes.

1.3. Chronic rejection

Acute allograft rejection has been largely controlled, with advances in modern immunosuppression. Long term survival of grafts is hampered by a slow deterioration in

graft function, leading to eventual failure. This process, loosely termed chronic rejection, is characterised by the development of chronic allograft vasculopathy. The term chronic rejection was introduced by Hildemann in 1960 referring to rejection of skin grafts observed in animal models (Hildemann and Walford, 1960). They reported that some of the grafts in their study had delayed onsets of rejection, with prolonged periods of rejection accompanied by atrophy and fibrosis. During this process, the graft arteries developed intimal hyperplasic lesions that, by obstructing blood flow to the grafts, resulted in relative tissue ischaemia. This is known by various names in the literature, but will be referred to as allograft vasculopathy (AV) throughout this thesis. Allograft vasculopathy has an incidence of 8% within the first year of cardiac transplants, 32% within five years, and 43% within first eight years (Taylor et al., 2006). One study has even detected AV in almost 50% of cardiac allografts within the first year after transplantation (Tuzcu et al., 2005). The development of AV is associated with high recipient morbidity and mortality (Taylor et al., 2006; Tuzcu et al., 2005). Similarly, chronic rejection accounts for 40% of renal graft losses beyond the first six months following transplantation (Li and Yang, 2009).

AV is characterised by diffuse, progressive and concentric narrowing of the arteries as a result of formation of neointima (Billingham, 1992). The neointima consists predominantly of smooth muscle cells and associated extracellular matrix, fibroblast cells, macrophages and T cells. Initially, smooth muscle cells from media were thought to be the origin of these smooth muscle cells; but at least two other sources of these have been identified. This includes recipient smooth muscle cells as well as donor or recipient endothelial cells (Hillebrands et al., 2001), which can differentiate into mesenchymal or smooth muscle cells under the influence of cytokines such as TGFβ (Zeisberg et al., 2007). The internal and external lamina remains intact. The media layer is mostly unaffected. However, AV results in the development of adventitial fibrosis (Libby and Pober, 2001) which may contribute to the vessel contraction independent of intimal proliferation. Adventitia provides the vasomotor tone to the arteries; adventitial fibrosis will thus limit the outward expansion of arteries to compensate for the thickened neointima (Mitchell and Libby, 2007). As a result, the lumen of the arteries is narrowed, leading to reduced blood flow to the grafts and ischemic parenchymal atrophy in the grafts.

Both AV and atherosclerotic diseases are atheromatous in origin, sharing many common characteristics like endothelial dysfunction, intimal thickening, aberrant buildup of extracellular matrix and leukocyte infiltration. But the pathological features of AV differ from that of atherosclerosis, in that AV is a diffuse and concentric plaque deposition in all vessel types within the allograft, mostly intra-myocardial vessels (Billingham, 1992; Lin et al., 1994), whereas atherosclerosis is focal and eccentric and involves proximal coronary vessels. Signs of inflammation are seen in AV but rarely in atherosclerosis. Furthermore in atherosclerosis, there is deposition of calcium and disruption of internal elastic lamina, which is not observed in AV (Aranda and Hill, 2000). The development of AV can occur within a year (Weis and von Scheidt, 2000) compared to atherosclerosis which evolves over decades (Stary, 1989).

1.3.1. Pathophysiology of allograft vasculopathy

The pathophysiologic features of AV, although not completely understood, likely involve components of both immune-mediated and nonimmune-mediated endothelial damage (Gao et al., 1988). There is substantial evidence that an interaction between alloantigen dependent alloimmune responses (adaptive and innate immune responses) and alloantigen independent factors (donor and recipient characteristics, ischaemic reperfusion injury (IRI)) contributes to the development of chronic rejection. The cumulative endothelial injury is thought to trigger a cascade of repair mechanisms that involve vascular cell proliferation, fibrosis and vascular remodelling, ultimately leading to intimal thickening and luminal narrowing (Hosenpud et al., 1992). The endothelial injury in AV can be denuding or non-denuding. In non-denuding injury, a rapid replacement of injured endothelial cells leads to endothelial dysfunction (Russell, 2000). Both immune-related and nonimmune-related factors contribute to non-denuding injury. In contrast, denuding injury is caused by ischemia–reperfusion injury, which leads to exposure of underlying smooth muscle cells and extra cellular matrix to blood, thus raising the potential for thrombus formation. Although thrombus formation is not an obvious feature of AV, it can trigger the clotting cascade, which has been shown to contribute to the development of arterial disease (Yen et al., 2002).

1.3.1.1. Alloantigen dependent alloimmune responses

The trigger to cell-mediated rejection is allorecognition, in which non-self-antigens are detected by the host immune system (Rogers and Lechler, 2001). These non-self alloantigens are recognised by the host immune system by three distinct mechanisms called direct, indirect and semi-direct pathways. The direct pathway results from the recognition of foreign intact major histocompatibility (MHC) molecules on the surface of donor APCs by recipient T cells (Rogers and Lechler, 2001). Direct alloimmunity is thought to be short lived, because donor APC are destroyed during the initial priming of T cells (Saiki et al., 2001). In support, direct alloreactivity was not detected in renal transplant recipients suffering chronic rejection (Baker et al., 2001a; Baker et al., 2001b). Indirect allorecognition occurs when donor antigens are internalized, processed, and presented as peptides by host APCs in the context of self-MHC II to recipient CD4 T cells. Because indirect pathway responses can theoretically occur for months or years after transplant, they are thought to be more relevant for the development of chronic rejection or AV (Conlon et al., 2012; Rogers and Lechler, 2001; Weis and von Scheidt, 2000). In the semi-direct pathway (Smyth et al., 2006), recipient DCs not only acquire donor MHC, but also re-present it as intact (non-processed) antigen on the cell surface to be recognized by recipient T cells (Marino et al., 2016). However its contribution to AV is unknown.

CD4 T cell activation is central to the pathogenesis of AV. In animal models, blocking CD4 T cell co-stimulation achieves graft tolerance and significantly reduces vasculopathy (Fischbein et al., 2000; Glysing-Jensen et al., 1997). Clinical studies show a predominance of Th-1 type immune responses in AV (Methe et al., 2005a). Similarly increased Th-1 type immune responses both in blood and biopsy samples have been associated with chronic rejection (Methe et al., 2005b; van Besouw et al., 1997). The importance of T cells is highlighted by the fact that nude mice (that lack T cells, but contain a mature B cell compartment) do not develop AV, even when there is a major MHC mismatch between donor and recipient (Shi et al., 1996). Similarly, treating recipients with anti CD4 and anti CD8 monoclonal antibody ameliorates AV in donor hearts (Nagano et al., 1998). Activated T cells not only cause injury to the graft endothelium via cytokines such as IFN-γ (Koh et al., 2004), they also provide essential help to B cells for the generation of humoral immunity (Steele et al., 1996; Taylor et al., 2007).

Although research has tended to focus upon cellular immunity as central to the pathogenesis of AV; increasing evidence suggests that humoral alloimmunity plays an important role (Vongwiwatana et al., 2003). The development of anti-donor HLA alloantibody after kidney transplantation is now known to be a major determinant of graft survival (Gaston et al., 2010). The de novo development of donor HLA specific antibodies is linked to the development of post-transplant glomerulopathy, which is associated with a reduction of 10 years in median graft survival (Eng et al., 2011). Similarly, alloantibody development correlates with early heart (Ho et al., 2011), lung (Girnita et al., 2007) and liver (Stegall, 2010) transplant failure. Antibodies target endothelial cells and can mediate injury by complement-dependent and independent mechanisms. The role of antibody mediated rejection (AMR) in the pathogenesis of AV remained controversial until 1990, when Feucht et al reported C4d deposition, a durable marker of complement activation, in renal graft capillaries (Feucht et al., 1991). Later it was confirmed that C4d staining was associated with the presence of donor specific antibodies in acutely rejecting renal grafts (Collins et al., 1999). Many reports have suggested an association between C4d deposition and histopathologic features of chronic rejection in renal grafts (Regele et al., 2002). As the presence of circulating anti-HLA alloantibodies often correlates with histological changes of chronic rejection within the allograft (Mauiyyedi and Colvin, 2002; Regele et al., 2002) and is associated with early graft failure (Lee et al., 2002), the combination of donor specific antibody (DSA) and C4d deposition has been accepted as a reliable criteria for diagnosis of AMR (Rodriguez et al., 2005; Smith et al., 2005). Complement activation leads to graft injury by producing leukocyte chemoattractant products C3a and C5a and by lysing endothelial cells through membrane attack complex C6, C7, C8 and C9 (Nakashima et al., 2002).

1.3.1.2. Interaction between alloantigen independent factors and alloimmune responses

Allograft Vasculopathy (AV) has long been regarded as synonymous with chronic rejection, but this is misleading, as it does not adequately take into account alloantigen independent factors. Alternatively, the 'response to injury' concept may provide a better model of the underlying factors and their interactions that lead to the development of AV.

This concept states that graft vasculopathy is the result of a continuous inflammatory response to tissue injury initiated by both alloantigen-dependent and independent stress responses (Vassalli et al., 2003; Weis and von Scheidt, 2000).

A variety of nonalloimmune insults, including brain death, organ preservation, surgical trauma, ischemia-reperfusion injury, cytomegalovirus (CMV) infection, hypertension, hyperlipidaemia, have been associated with immune activation, endothelial dysfunction, and development of AV (Rahmani et al., 2006; Sharples et al., 2003; Valantine, 2003; Vassalli et al., 2003; Weis et al., 2004; Weis and von Scheidt, 2000). Upon endothelial activation, endothelial adhesion molecule and chemokine expression is upregulated, vascular growth factors and thrombogenic molecules are expressed, and immune cells invade the graft (Rahmani et al., 2006; Valantine, 2003; Weis and von Scheidt, 2000). One good example of how increased graft immunogenicity induces a host alloimmune response, even in the absence of non-self-antigens, is brain death–induced immune activation. In brain death donors, a series of neural, hormonal and molecular changes (Pratschke et al., 2005) occur that result in upregulation of adhesion ligands and MHC molecules, as well as increased cytokine secretion. These changes can trigger T cell activation via the different allorecognition pathways. Nonspecific inflammation, derived from ischaemia-reperfusion injury or metabolic dysregulation, further promotes the shedding of intact, soluble MHC molecules, which again might prime the indirect allorecognition pathway (Pratschke et al., 2005).

1.4. Autoimmunity and Allograft vasculopathy

Although several alloimmune dependent and alloimmune independent factors contribute to the development of AV against donor derived MHC antigens, the role of antibodies against non-HLA donor antigens has been increasingly associated with poor graft outcomes (Dave and Bayless, 2014; Dragun et al., 2013; Dragun et al., 2016; Dragun et al., 2005; Fedoseyeva et al., 2002; Fedoseyeva et al., 1996; Fedoseyeva et al., 1999; Mahesh et al., 2007; Zhang and Reed, 2016). These non-HLA antigens are expressed mostly on epithelial and endothelial cells. MHC class I chain-related gene A (MICA) and tissue specific autoantigens such as vimentin, cardiac myosin (CM) and collagen V are the most studied non-HLA transplant antigens. The impact of these non-HLA antibodies is most dramatically demonstrated by the acute AMR that occurs in about 3% of cases in the absence of demonstrable anti-HLA alloantibody (Amico et al., 2008). Nevertheless, their contribution to chronic rejection remains controversial, and it is possible that they either represent bystander responses that are triggered as a consequence of ongoing, aggressive alloimmune

responses (Fukami et al., 2009) or that they may affect rejection by precipitating or augmenting conventional T and B cell alloimmune responses towards the graft (Cardinal et al., 2013; Harper et al., 2016; Lovegrove et al., 2001; Yoshida et al., 2006). Fukami et al demonstrated that antibodies to donor-MHC class I can induce autoimmunity, mediated by IL-17, which plays a pivotal role in chronic rejection post-lung transplantation. His work involved intra bronchial administration of anti-MHC class I antibodies into native Balb/c mice lungs. Ligation of MHC class I molecules of the lung parenchyma by specific anti-MHC class I antibodies resulted in cellular infiltrates, endotheliitis, hyperplasia and fibrosis around vessels and bronchioles. This led to the occlusion of distal bronchial airways similar to chronic rejection following human lung transplantation. Lungs of recipient mice of anti-MHC class I antibody demonstrated induction of IL-17 which resulted in the de-novo development of antibodies to self-antigens, K-α1 tubulin and collagen V. Interestingly, IL-17 neutralization by anti-IL-17 resulted in reduction of autoantibody and lesions induced by anti-MHC class I antibodies. Harper et al work (Harper et al., 2016) demonstrated that graft versus host recognition by donor CD4 T cells augmented the recipient cellular and humoral alloimmunity in BL6 recipients of bm12Kd.IE cardiac allografts. This augmentation of alloimmune response in the recipients resulted in more severe allograft vasculopathy and early graft failure. Recent evidence from heart and lung transplant recipients suggests, however, that the responses directed against several tissue specific self-proteins may contribute directly to chronic rejection (Burlingham et al., 2007; Goers et al., 2008; Jurcevic et al., 2001; Kalache et al., 2011). The strongest clinical evidence comes from lung transplant recipients (Burlingham et al., 2007), in which peripheral blood mononuclear cell (PBMC) responses to collagen II and collagen V were monitored prospectively in recipients using a trans-vivo delayed type hypersensitivity (TV-DTH) assay. They found that PBMCs from lung transplant recipients were frequently collagen V reactive, whereas collagen V reactivity was not detected in either normal healthy controls or kidney transplant recipients with goodpasture's syndrome who were reactive to collagen IV. Furthermore, the highest relative risk of bronchiolitis obliterans (BOS) development was observed in patients with a positive response to collagen V. In a separate study, Kalache et al (Kalache et al., 2011) investigated the relationship between AV and anti-CM autoimmunity by performing a cross-sectional study of 72 heart transplant recipients (40 with AV and 32 without). In a multivariate analysis, they found that PBMC from patients with AV responded more frequently to, and to a broader array of, CM-

derived peptides than those without AV. Furthermore, in their cohort, detection of either CM-peptide-reactive T cells or anti-CM antibodies was highly, and independently, indicative of development of AV. Fedoseyeva et al further demonstrated that in a murine model, modulating the T cell response to cardiac myosin self-protein resulted either in accelerated rejection, or indefinite heart graft survival (Fedoseyeva et al., 2002; Fedoseyeva et al., 1999).

These studies have, however focused on T lymphocyte autoimmune responses; how the development of autoantibody (as is typically described in human studies) contributes to graft rejection is still not clear.

1.4.1. Autoantibody formation following transplantation

Failure of the central mechanisms of clonal deletion and receptor editing to eradicate all self-reactive B cells results in the presence of autoreactive B cells in the periphery which are readily found in normal individuals (Wardemann et al., 2003). In the periphery, they are likely to encounter target autoantigen, and thus peripheral mechanisms must also exist to keep these autoreactive B cells in a quiescent stage (discussed in section 1.2.1). One of the main mechanism is through control of the availability of T cell help to these autoreactive B cells. In transplant recipients humoral responses are long lasting and result in the development of high affinity antibodies which are somatically mutated and class switched. These LL humoral responses are essentially the output of germinal centres which are ultimately dependent on the provision of help from CD4 T cell from follicular helper CD4 T cells (T_{FH}) cells (discussed in 1.5). T_{FH} cell recognise antigen peptide-MHC complex of the B cells with high affinity and result in the development of GC response. With recent work in autoimmunity, availability of help to autoreactive B cells from T_{FH} cells has now been confirmed (King C, et al, 2008; Linterman MA, 2009) that supports the earlier findings that provision of T-cell help can itself trigger autoimmunity (Guay HM, 2007; Cook MC, 1998; Fulcher DA, 1996; Seo SJ, 2002; Keech CL, 2001).

Several mechanisms have been proposed to explain how T-cell self- tolerance is broken to supply critical help for production of autoantibody. Tissue destruction from, for example, an infectious inflammatory process, may break anergy in the peripheral autoreactive T cells through presentation of target epitopes at much greater frequency than in the resting stage (Anderton and Wraith, 2002). In addition, inflammation may lead to the generation and

presentation of new cryptic T cell epitopes; either through release of self-proteins that are normally sequestered and unavailable for immune recognition, or alteration in protease activity within the APC (Manoury et al., 2002). Similarly, neoantigens may be formed through alterations in protein structure from posttranslational modification (Anderton, 2004; Doyle and Mamula, 2012) or oxidative stress (Rasheed et al., 2006). It is also possible that a subset of T cells responding to the infectious agent may provide help through cross-reactive recognition of autopeptide epitope presented by autoreactive B cells (Marrack et al., 2001).

1.4.2. What is the target of this autoantibody within the allograft?

Very little is known about the precise targets of the autoantibody responses that are thought to contribute to development of AV. This is a major obstacle in postulating for how circulating autoantibody contributes to vasculopathy through direct binding to allograft endothelial cells (ECs) as majority of target autoantigens are intracellular and are not exposed on the cell surface. Nevertheless, intracellular proteins can be translocated as apoptotic blebs on the cell surface during apoptosis like caspase dependent cleavage of vimentin leads to its surface expression. Hence, autoantibody binding to the exposed autoantigens will not occur until they are exposed by endothelial damage triggered by innate and adaptive alloimmune responses (Fukami et al., 2009). This may also explain why transplant associated autoimmunity appear to damage predominately the allograft, rather than native organs (Benichou et al., 2007; Harper et al., 2016; Mahesh et al., 2007; Win et al., 2009). The expression of autoantigens on the apoptotic cells only raises further question that how autoantibody binding to these cells would mediate progression of allograft vasculopathy. However, certain autoantigens may also be expressed on the healthy EC like intercellular adhesion molecule-1 (ICAM-1). Antibodies to Anti-ICAM-1 may contribute to the endothelial cell activation by binding to the endothelium, causing activation of proinflammatory signalling pathways (Lawson et al., 2005).

The other potential alternative may be that circulating autoantibody may be internalized through cell surface receptors (some of the identified receptors include myosin and calreticulin) and then bind to intracellular autoantigens within the cells (Yung and Chan, 2008). A final possibility is that the impact of autoantibody on endothelial signalling is

mainly paracrine effect. The binding of autoantibody to other cells like leukocytes in the immediate vicinity leads to activation of endothelial activation (Leong et al., 2008).

1.5.3. Mechanisms for the development of allograft vasculopathy following autoantibody binding

There is paucity of information about the mechanisms how autoantibody contributes to AV. This is mainly due to the fact that vasculopathy has complex pathophysiology and the factors that initiate mononuclear cell infiltration leading to intimal smooth muscle proliferation and eventual fibrosis, remain largely unknown. However, there are two potential mechanisms by which autoantibody might be contributing to AV; firstly by directly binding to the healthy endothelial cells and secondly by binding to autoantigens exposed only as a result of EC death.

1.5.3.1. Direct effects of bound autoantibody on healthy endothelial cells for the development of allograft vasculopathy

The evidence for direct effects of bound autoantibody on healthy endothelial cell is provided by Bordon et al work in which incubation of endothelial cells with antiendothelial cell antibodies (AECA) can induce apoptosis of endothelial cells (Bordron et al., 1998). In this study, AECA positive sera from systemic sclerosis (SS) patients was compared with the controls with other diseases. AECA derived sera from six of the eight patients with SS led to the expression of phosphatidylserine (PS) on the surface of endothelial cells which was significantly more frequent in the SS group when compared to the control group. This surface expression of PS preceded other events associated with apoptosis including DNA fragmentation. This occurred independent of complement fixation as apoptosis-inducing anti-endothelial antibodies did not recognize the Fas receptor. Macrophage and natural killer cell can recognise the bound autoantibody to EC, this recognition can also trigger EC death by antibody dependent cell mediated cytotoxicity. Although there is limited evidence for the role of antibody dependent cell mediated cytotoxicity in autoimmunity, some murine transplant studies have suggested contribution of NK cells towards development of AV (Graham et al., 2009; Uehara et al., 2005). Apoptosis may seem an integral part to the development of AV, in that exaggerated healing process which it triggers may ultimately result in fibroproliferation.

Autoantibody binding would also potentially result in activation of complement components, ultimately leading to formation of membrane attack complex (MAC). This

MAC will kill target EC through necrosis, but apoptosis may also takes place. Early apoptotic cells are not only opsonized by complement components but may also be lysed raising the possibility that under certain conditions apoptosis may be associated with an inflammatory reaction (Fishelson et al., 2001). This inflammatory milieu may trigger the intracellular signalling cascade and leads to AV development. Although incubation of rat lung epithelium with collagen V positive immune sera results in complement-dependent cytotoxic killing, only a small number of murine studies suggest complement deposition in association with a vasculopathy that is presumably autoantibody mediated (Azimzadeh et al., 2005; Win et al., 2009).

Apart from cell lysis, intracellular signaling cascades can activate the EC. Membrane attack complex may play a crucial role in the formation of pores within EC membranes that then can trigger IL-1α signalling pathway. This down signalling cascade activation then leads to upregulation of inflammatory adhesion molecules and pro-coagulant mediators on the activated ECs. Similarly there is widespread expression of receptors for C3a and C5a on ECs (Gasque et al., 1997) and ligation of these receptors by C3a and C5a respectively after activation of complement system by bound autoantibody can trigger diverse responses including actin polymerization, expression of adhesion molecules and release of von Willebrand factor (Brunn et al., 2006; Saadi et al., 2000). Despite all this, there is no formal demonstration of progression of EC activation to development of vasculopathy and how such signalling cascades trigger the development of fibroproliferative responses is not clear. However, the informative evidence for the role of intracellular signalling in development of AV is provided by various studies in which anti-MHC class I alloantibody responses were examined on endothelium (Jin et al., 2005; Jin et al., 2007; Jindra et al., 2008; Zhang and Reed, 2009). Anti-MHC class I alloantibody triggers various signalling cascades including tyrosine phosphorylation of focal adhesion kinases, upregulation of Rho proteins and activation of Mammalian target of rapamycin (mTOR) pathways (Coupel et al., 2004). Focal adhesion kinases and Rho family proteins are critical for actin fibre formation and cytoskeleton reorganization and interestingly blockage of Rho kinase pathway in a murine model of heart transplant blocked the development of AV (Hattori et al., 2004). Conceptually it is quite possible for MHC class I alloantibody to activate signalling cascade as being a membrane protein whose main function is to conduct information across the

membrane, however whether similar transduction can take place with autoantigens that lack a well-defined signalling pathway is not known and the evidence for this is sparse in autoimmunity and transplantation (Dragun et al., 2005; Holmen et al., 2007; Lawson et al., 2005).

1.6.3.2. Indirect effects of autoantibody binding to autoantigens for development of allograft vasculopathy following

Autoantibody binding to apoptotic ECs can lead to endothelial activation which is widespread. This in turn can trigger the complement components. Activated complement mediators then potentially can affect all the ECs in the vicinity, which is a nonspecific phenomenon, irrespective of their binding to apoptotic ECs. Similarly, the fibroproliferative process generated by apoptosis are paracrine effect which may get augmented by autoantibody binding to the autoantigens that have translocated to the surface of apoptotic endothelial cells. It is quite possible that the humoral autoimmunity may be contributing to AV by different mechanisms, different from alloimmune responses following transplantation. In support, the binding of antivimentin autoantibody to circulating leukocytes results in release of platelet activating factor and prothrombotic factors which exert a paracrine activating effect on platelets (Leong et al., 2008). Although, thrombosis of vessels leading to their occlusion is not a characteristic of AV, clotting process has been implicated in its pathogenesis. In one of the study, tissue plasminogen activator (tPA) levels were measured in the arteriolar smooth muscle cells of allograft biopsies. This demonstrated that the allografts with low levels of t-PA had significantly higher AV and poor allograft survival compared to the group with normal levels of t-PA (Labarrere et al., 1995). Humoral autoimmunity may contribute to AV by augmenting recipient alloimmune responses (Harper et al., 2016). Inflammatory milieu which is produced by autoantibodies can lead to nonspecific activation of donor and recipient DCs which may be responsible for priming of allospecific lymphocytes. Ligation of receptors for various activated complement meditators on the surface of T cells and APCs within the allograft have been described as one of the regulators of T cell proliferation and survival (Heeger et al., 2005; Peng et al., 2008; Strainic et al., 2008; Zhou et al., 2007). Although activation of naïve alloreactive T cells take place in secondary lymphoid organs, local complement mediators may augment the ability of the primed CD8 T cells to respond to the allogenic endothelium (Raedler et al.,

2009). Finally, spreading T and B lymphocyte responses from auto- to allo-antigens may contribute to progression of AV. There is a small fraction of autoreactive B cells which escape central deletion by receptor editing and still continue to express autoreactive receptors in the periphery, but at low levels (Gerdes and Wabl, 2004; Liu et al., 2005). Such dual positive autoreactive B cells may will possess principal Ig receptor specific for alloantigen. However, their ability to present allopeptide may greatly be augmented after ligation of their second autoreactive BCR (Ciubotariu et al., 1998; Suciu-Foca et al., 1998).

1.5. Germinal centre responses and transplantation

Most of our understanding of the germinal centre response is the result of the studies of murine responses to model protein antigen, and only a limited number of studies have examined germinal centre alloimmunity in transplantation (Chhabra et al., 2018; Conlon et al., 2012; Kim et al., 2014). Given that the humoral responses generated following transplantation are typically long lived and therefore likely mediated by bone-marrow resident long-lived plasma cells further evaluation of the allospecific GC response is an important area for further study, particularly because the development of anti-donor HLA alloantibody after transplantation has been unequivocally correlated with poorer late graft outcomes (Gaston et al., 2010). A greater understanding of the mechanisms that lead to the production of long lasting plasma cells (LLPC) and memory B cells in clinical transplantation is likely to inform development of strategies that selectively target the humoral alloimmune response as a means of improving long-term allograft survival.

The autoantibody responses that have been increasingly emphasised in chronic rejection of solid organ transplants also appear to be long-lasting, and presumably, are similarly the result of a germinal centre reaction. However, an independent role for the autoreactive germinal centre response in the progression of allograft vasculopathy (AV) has not yet been shown. Confirmation that autoreactive germinal centres responses can cause graft damage is therefore an important consideration that needs to be first addressed.

1.6. Experimental model and aims

GC responses to model protein antigen have been examined in depth in the last decade, but not so far in relation to organ transplantation. Hence in this thesis, the development of germinal centres was mainly investigated in bm12 to BL6 model. The bm12 strain has a mutant form of I-A that differs by three amino acids from the I-A^b β-chain of BL6 strain. This has been (Ford et al., 2002; Hori et al., 1992; Kitagawa et al., 1990; Yuan et al., 2008), and continues to be (Lei et al., 2016; Win et al., 2009; Zhou et al., 2015), one of the most widely used models of chronic rejection, with several seminal manuscripts published in top line journals. In addition, it has long been used as a model of murine lupus (Eisenberg and Via, 2012). Nevertheless, this model remains relevant as a model for clinical transplantation for the following reasons:

- The recipient and donor are minimally MHC mismatched. This resembles clinical kidney transplantation, where organs are typically allocated to recipients on the basis of close HLA matching.
- We have shown that manipulation of the donor CD4 T cell population prior to transplantation impacts upon graft outcomes (Win et al., 2009). This has immediate relevance to the recent introduction into clinical practice of *ex vivo* perfusion of organs after retrieval, but prior to transplantation. Such strategies are likely to alter the resident CD4 T cell population within the allograft.

In this model, we have shown that development of IgG antinuclear autoantibody following transplantation is dependent on the presence of passenger CD4 T cells in the donor hearts (Win et al., 2009). Histopathological examination of the transplanted donor hearts revealed endothelial complement deposition, which was highly suggestive of humoral vascular damage to the allografts. These features were absent, and vasculopathy was minimal, in heart grafts from B cell deficient recipients; conversely hearts grafts in recipients primed for autoantibody generation prior to transplant developed severe AV and were rejected rapidly. From the above work (Win et al., 2009), we concluded that the development of autoantibody in the recipient was dependent on the presence of passenger donor CD4 T cells in allograft, however, role of recipient CD4 T cells was not investigated. Although cGVH model does not propose any essential role for the recipient's endogenous T cells, some murine transgenic GVH models have suggested that recipient T cells may be involved in the

development of SLE like autoimmune syndrome (Chen et al., 1998; Choudhury et al., 2005; Gonzalez et al., 1995; Rolink et al., 1983). Nevertheless, no definite role has been identified in bm12 to B6 model. Hence, further exploration was needed to ascertain the role of recipient CD4 T cells in this model.

The aims of this thesis are as follows:

1. To study the interaction between donor and recipient CD4 T cells with recipient B cells in the initiation and maintenance of autoantibody responses following bm12 heart transplantation.
2. To investigate the role of germinal centre autoantibody responses following solid organ transplantation in progression of allograft vasculopathy.

2. Methods

2.1. Animal related work

2.1.1. Animal husbandry

C57BL/6 (BL6, H-2^b) [wild-type (WT)] and BALB/c (H-2^d) mice were purchased from Charles River Laboratories (Harlow, UK); all other animals were bred and maintained in specific-pathogen free facilities at Central Biomedical Services, University of Cambridge. Animals were cared for and used in accordance with the Animals (Scientific Procedures) Act 1986.

Details of the different strains of mice, together with their H2 haplotype, are provided in Table 2.1. Donor and recipients were sex matched.

Sh2d1a$^{-/-}$ BL6 mice (SAP^{-ve}.BL6) were gifted from Michelle Linterman (University of Cambridge, UK) (Linterman et al., 2009). SAP^{-ve}.BL6 mice have defective T_{FH} CD4 T cell population and hence unable to form germinal centres The SAP gene (*Sh2d1a*$^{-/-}$) is X-linked and male SAP^{-ve} animals were not able to breed with SAP$^{-/-}$ females; hence SAP$^{+/-}$ heterozygous female was crossed with a WT BL6 male. The offspring were genotyped according to the protocol discussed in section (2.2.10) and only SAP^{-ve}.BL6 males were used in experiments. In addition heterozygous female SAP$^{+/-}$ were crossed with male bm12 mice to produce SAP^{-ve} on the bm12 background. For bm12, homozygosity was achieved by backcrossing bm12.SAP$^{+/-}$ heterozygous females with a homozygous male bm12. Bm12 status was confirmed by genotyping by identifying the presence of the bm12 band and absence of the BL6 WT band on gel electrophoresis of the PCR product (discussed in section 2.2.10).

Similarly, for SAP^{-ve} TCR75 animals, male TCR75 mice were crossed with heterozygous SAP$^{+/-}$ BL6 females and from the offspring, SAP$^{+/-}$ TCR75$^{+/-}$ RAG$^{+/-}$ females were back-crossed with male TCR75$^{+/-}$RAG$^{-/-}$SAP$^{+/+}$ in order to generate TCR75$^{-/-}$SAP^{-ve} RAG$^{-/-}$ mice on a RAG background. TCR75 status was assessed by phenotyping for Vß8.3 chain and CD4 and RAG status was assessed by absence of B cells. As mentioned above, only male SAP^{-ve} bm12 and male SAP^{-ve} TCR75 mice were used in experiments.

Table 2.1. Mouse strains with their MHC and genetic modifications

Mouse strains	Description	MHC	T cells	B cells	Genetic Modification
BL6	Wild type H-2^b	K^b D^b $I\text{-}A^b$ $I\text{-}E^{-ve}$	WT	WT	N/A
BALB/c	H-2^d	K^d D^d L^d $I\text{-}A^d$ $I\text{-}E^d$	WT	WT	N/A
bm12	BL6 natural mutant	K^b D^b $I\text{-}A^{bm12}$ $I\text{-}E^{-ve}$	WT	WT	N/A
TCR$^{bd-/-}$ BL6	TCR deficient on BL6 background	K^b D^b $I\text{-}A^b$ $I\text{-}E^{-ve}$	Absent	WT	Knock out for β and δ chains for T cell receptors
bm12TCR$^{bd-/-}$	TCR deficient on bm12 background	K^b D^b $I\text{-}A^{bm12}$ $I\text{-}E^{-ve}$	Absent	WT	Knock out for β and δ chains for T cell receptors on bm12 background
bm12.Kd	Cross of bm12 with BL6.K^d	K^d K^b D^d $I\text{-}A^{bm12}$	Wild type	WT	
CD45.1 BL6	Wild type H-2^b	K^b D^b $I\text{-}A^b$ $I\text{-}E^{-ve}$	WT	WT	Transgenic for congenic marker which is CD45.1 in this animal
RAG2$^{-/-}$ BL6	Recombinase activating gene 2KO	K^b D^b $I\text{-}A^b$ $I\text{-}E^{-ve}$	Absent	Absent	Deletion of *Rag2* gene

TCR75	TCR75 BL6 on *Rag* background	K^b D^b $I\text{-}A^b$	Monoclonal; specific for $I\text{-}A^b$ restricted $H2\text{-}K^d{}_{54\text{-}68}$	Absent	Insertion of transgene for TCR Vα1.1/Vβ8.3 under CD1 promoter Deletion of *Rag1* gene
KdTCR$^{bd-/-}$	BL6.Kd was crossed with $TCR^{bd-/-}$	K^d K^b D^b $I\text{-}A^b$	Absent	WT	Addition of additional Kd transgene to $TCR^{bd-/-}$
DM$^{-/-}$BL6	DM knockout	K^b D^b $I\text{-}A^b$ $I\text{-}E^{-ve}$	WT	Lack DMA molecule, confirmed on genotyping	Deletion of DMA molecule on all antigen presenting cells
SAP$^{-/-}$ BL6	SAP knockout	K^b D^b $I\text{-}A^b$ $I\text{-}E^{-ve}$	Have CD4 T cells but cannot differentiate into T_{FH} cells	WT	Targeted mutation on *SH2D1A*/*SAP gene*
SAP$^{-/-}$bm12	SAP knockout on bm12 background	K^b D^b $I\text{-}A^{bm12}$ $I\text{-}E^{-ve}$	Have CD4 T cells but cannot differentiate into T_{FH} cells	WT	Targeted mutation on *SH2D1A*/*SAP gene*
SAP$^{-/-}$TCR75	TCR75 BL6 with additional SAP molecule deletion	K^b D^b $I\text{-}A^b$	Monoclonal; specific for $I\text{-}A^b$ restricted $H2\text{-}K^d{}_{54\text{-}68}$ but T_{FH} cells defective function	WT	Targeted mutation on *SH2D1A*/*SAP gene*

2.1.2. Surgical procedures

All surgical procedures were carried out under 10-40x magnifications using a light microscope (Carl Zeiss OPM11-FC Thornwood, NY, USA). Anaesthesia was induced and maintained by inhalation of 1-2% isofluorane (Abott Laboratories Ltd, UK). Sterile conditions were observed throughout the procedures.

2.1.2.1. Heterotopic heart transplantation

Background

Vascularised cardiac grafts were transplanted intra-abdominally by the technique of Corry et al(Corry et al., 1973). Briefly, the donor aorta was anastomosed to recipient abdominal aorta and donor pulmonary artery was anastomosed to recipient IVC. Blood enters the recipient aorta and after perfusing the coronary circulation at the coronary ostia returns to the right atrium. Then blood is pumped out to the recipient IVC (Figure 2.1)(Hasegawa et al., 2007)

Donor operation

The abdomen was opened with a midline incision. The skin and abdominal wall were retracted with a self-retaining retractor. The small bowel was dissected and retracted to expose the abdominal aorta and IVC; then 100µl Heparin Sodium at 250units/ml (i.e. approximately 0.5mg/ml) (Wockhardt, Wrexham, UK) was injected into the vena cava. The abdominal aorta and vena cava were then divided and the donor animal exsanguinated. Thoracotomy was performed and the thymus removed. The heart was cooled in situ by irrigation with 4°C saline. The inferior vena cava, hemiazygos vein and superior vena cava were ligated and divided using 7/0 silk suture (Pearsalls Ltd, Somerset, UK). The ascending aorta was transacted proximal to the origin of the right brachiocephalic artery and the main pulmonary artery was transacted proximal to the division into right and left pulmonary arteries. A single 7/0 suture was tied around the base of the heart, ligating the branches of the pulmonary veins. The heart was removed, gently compressed to expel blood and placed in 4°C sterile saline until re-anastomosis.

Recipient operation

Recipients were given 100µl Temgesic (Reckitt Benckiser Healthcare, Hull, UK) at 100µl/ml in phosphate buffered solution (PBS; OXOID, Hampshire, UK) by subcutaneous injection. The

abdomen was opened with a midline incision and skin and abdominal wall retracted. To expose the retroperitoneum, the small bowel was gently dissected and retracted superiorly and left on the chest of the recipient covered in wet and warm saline gauze. Blunt dissection of the retroperitoneum revealed the infra-renal abdominal aorta and IVC. Any lumbar vessels were ligated with a single 7/0 suture. Two microsurgical clips were applied across both the aorta and IVC, firstly inferior to the renal arteries and secondly superior to the division of the IVC. The donor heart was removed from cold saline and placed in the recipient abdomen. An aortotomy was performed and the donor aorta sutured to recipient aorta end-to-side using continuous 10/0 proline sutures (Bear Medic, Tokyo, Japan). A venotomy was performed and anastomosis of the donor pulmonary artery and recipient IVC performed using interrupted 10/0 proline sutures. Surgicell (absorbable cellulose, Johnson and Johnson Medical Ltd, UK) was lightly applied around the anastomosis to reduce bleeding and the clamps released. After the establishment of donor heart sinus rhythm, the abdominal wall and then skin were closed using 5/0 suture (Industrial & Scientific supplies Ltd, Linton, UK). The animal was placed in a 32°C incubator until recovery was complete.

It is important to note here that the donor lymphatic vessels are not anastomosed to the recipients and they may take upto 4 weeks to re-connect with the recipient lymphatic system after transplantation(Ruggiero et al., 1993). However during this period when the donor lymphatic vessels are disconnected, the priming of the alloimmune response takes place. For vascularized organs, with the disruption of lymphatics after transplantation, an alternative route of donor passenger leukocytes migration called reverse transmigration across capillaries has been described and donor passenger lymphocytes can be found in the spleens of recipient within 24hours of transplantation, hence spleen was used as a secondary lymphoid organ to study the humoral responses.

Monitoring graft function

Heart grafts behaved functionally as aorto-caval fistulae which were assessed by weekly abdominal palpation. Cessation of myocardial contractions was termed as rejection of the grafts and confirmed at the time of explantation of grafts. Grafts were explanted at predetermined time points after transplantation; half of the heart was embedded in OCT compound (VWR International, Lutterworth, UK) flash-frozen in liquid nitrogen and stored

at -80^0 C, and the other half was fixed in 10% formal saline and sent for EVG staining to determine the degree of luminal stenosis.

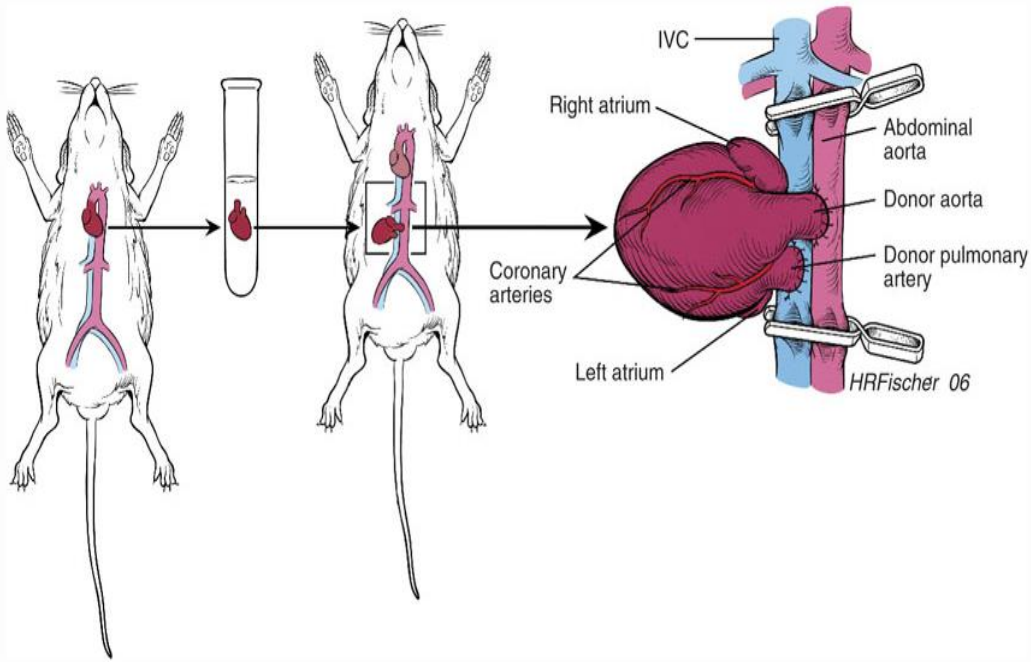


Figure 2.1. Mouse heterotopic heart transplant model.

The aorta and pulmonary artery of the donor heart are anastomosed to the recipient's abdominal aorta and inferior vena cava respectively; image from Hasegawa et al, permission granted from publisher Rights Link /Springer Nature (Hasegawa et al., 2007).

2.1.2.2. Skin grafts

Full thickness tail skin was obtained from sacrificed donor animals, cut into $1cm^2$ and placed in cold (4°C) saline. Recipient animal anaesthesia was induced and maintained with inhalational 1-2% isoflurane, subcutaneous analgesia was given and the animals placed prone on a heated (37°C) operating board. $1cm^2$ area of skin was removed from the upper dorsum of recipient and the donor skin was laid over the prepared area. The donor skin was sutured to the recipient's by 5/0 sutures, firstly in the four corners and then four stitches in between them. Rejection was defined as loss of skin graft at least two days post transplantation; loss of skin graft within 48 hours of grafting was defined as a technical failure.

2.1.3. Delivery of drugs, cells and antibodies

2.1.3.1. Intravenous (iv) injection

Where required, populations of purified lymphocytes or monoclonal antibodies were suspended in 100-200µl Hartmann's solution (Animal care Ltd, York, UK) and injected intravenously (iv) via the tail vein. Prior to injection, animals were placed in an incubator (39°C) for 10 minutes until veins were prominent.

2.1.3.2. Intraperitoneal (ip) injections

For some experiments, monoclonal antibodies were suspended in 300-500µl Hartmann's solution and injected intraperitoneally (ip) into anaesthetised recipients (anaesthesia induced and maintained by inhalational isoflurane).

2.1.3.3. Subcutaneous (sc) injections

When required, sc injection was given by anaesthetising the recipient by 2% isofluorane and skin was retracted on the dorsum of the recipient and immunogenic substances were injected along with complete Freund's adjuvant (CFA) or incomplete Freund's adjuvant (IFA).

2.1.4. Collection of blood and harvesting tissues

2.1.4.1. Collection of blood samples for serum

Approximately 20µl of peripheral blood was obtained through a superficial tail vein using a 25 gauge needle into a microvette (Sarstedt) from all the postoperative recipients or recipients of the purified lymphocytes after heating the animals in an incubator at 38°C for

10 minutes. The veins were compressed after collecting the blood until bleeding stopped. Where required, up to 1ml of blood was obtained at the time of sacrifice by direct cardiac puncture under terminal anaesthetic.

2.1.4.2. Collection of peripheral blood lymphocytes

Blood was collected in tubes pre-coated with heparin and processed as described in section below.

2.1.4.3. Harvesting organs and tissues and their storage

Organs or tissues were harvested from the experimental animals killed by cervical dislocation or under terminal anaesthesia. Spleens were collected in Roswell Park Memorial Institute 1640 tissue culture medium (Gibco^{TM}, Invitrogen, Paisley, UK) (RPMI) or embedded in OCT compound (VWR International, Lutterworth, UK), flash-frozen in liquid nitrogen and stored at -80°C and hearts were harvested and fixed in 10% formal saline.

2.1.5. In vivo depletion of CD4 T cells

CD4 T cells were depleted from the donors by three ip injections of 1.0mg of anti-CD4 mAb (YTS 191.1, hybridoma from the European Collection of Animal Cell Cultures) at six, five and 1 day prior to heart graft procurement. Depletion of CD4 T cells (typically >99%) (Motallebzadeh et al., 2012) was measured by flow cytometry of peripheral blood lymphocytes on the day before heart procurement and confirmed by flow cytometric analysis of donor splenocytes on the day of retrieval.

2.1.6. In vivo depletion of NK cells

PK 136 mouse hybridoma (anti-NK1.1 antibody) was purchased from ATCC (HB-191) and was grown and purified in house. Recipient mice were depleted of NK cell population by treatment with anti-NK1.1 antibody; animals received 500µg of antibody ip or iv on days -2, 0 and 2(Ali et al., 2016; Yu et al., 2006)

2.1.7. In vivo depletion of CD20 positive B cells

Murine anti-CD20 antibody was (250µg) injected to mice as per protocol of the experiment to deplete $CD20^{+}$ B cells (Hamaguchi et al., 2006).

2.1.8. Generation of bone-marrow chimeras

2.1.8.1. Generation of mixed chimerism

Mixed haematopoietic chimeras were set up using previously well described protocols, using a combination of depleting antibodies and total body irradiation (TBI)(Nikolic et al., 2010). Briefly, CD45.1 BL6 recipients were treated with anti-CD4 (YTS) on day -6 and -1 and anti-CD8 on day -1, 0, 1, and day 7. On day 0 recipients received 4Gy TBI and a single dose of 2mg of anti-CD154 mAb (MR1). Following conditioning on day 0 recipients also received an iv injection of $3x10^7$ purified bm12 bone marrow cells. Chimerism was confirmed by flow cytometric analysis of peripheral blood lymphocytes at least 4 weeks after reconstitution.

2.1.8.2. $RAG2^{-/-}DM^{-/-}$ bone-marrow chimeric mice

To generate $RAG2^{-/-}$. $DM^{-/-}$ bone-marrow chimeric mice, $RAG2^{-/-}$ mice were sublethally irradiated (2Gy) using a Caesium-137 source and reconstituted with 2 x 10^7 $DM^{-/-}$ bone marrow cells iv. Chimerism was confirmed by flow cytometric analysis of peripheral blood lymphocytes at least 4 weeks after reconstitution.

2.1.9. Preparation of hyperimmune sera for ELISA

To obtain antibody-containing serum for positive control for ELISA, animals were given a relevant immune challenge (table 2.2) followed by cardiac puncture under terminal anaesthesia 5 weeks post challenge.

Table 2.2. Generation of hyperimmune serum

Serum	Immune challenge	
	Recipient	Challenge
Anti-nuclear antibody	$TCR^{bd-/-}$	$5x10^6$ highly purified bm12 CD4 T cells
Anti-H2-K^d antibody	BL/6	BALB/c heart graft

2.2. Laboratory based procedures

All laboratory procedures were performed at the departments of Surgery, Medicine and Virology.

2.2.1. Cell preparation

All cell preparation was performed in a Microflow SE laminar flow hood (BIOQUELL, Andover, Hampshire, UK) with (RPMI) 1640 tissue culture medium (Gibco^{TM}, Invitrogen, Paisley, UK) referred to as 'medium', unless otherwise stated. Centrifugation was performed in a Howe 6K10 Centrifuge (Sigma Laboratory Centrifuges GmbH, Osterode am Harz, Germany) at 1200 revolutions per minute (rpm) for 7 minutes at 4°C, unless otherwise stated. Cells were counted with an Improved Neubauer counting chamber (Hawksley, Lancing, UK) under 20 x magnifications with a conventional table-top light microscope.

2.2.1.1. Splenocytes

Spleens were harvested, disaggregated and teased through a 40µm nylon cell strainer (Becton Dickinson, Franklin Lakes, NL, USA) using the rubber end of a 2 ml syringe plunger. The cells were washed with medium, centrifuged and resuspended and used as required by the protocol of the experiments.

2.2.1.2. Bone marrow cells

Media + 5% foetal calf serum (FCS) was used to flush bone marrow (BM) from the tibias and femurs of sacrificed animals. Single cell suspensions were obtained by teasing the BM through a 40 µm nylon cell strainer (as above). BMCs were washed with medium, centrifuged and resuspended.

2.2.1.3. Peripheral blood lymphocytes (PBLs)

Approximately 20µl of blood was collected as in Section 3.2.5 into a microvette containing approximately 5µl of unfractionated heparin. This heparinised blood was mixed with 200µl of 0.17M ammonium chloride for 5 minutes at room temperature to lyses erythrocytes and then centrifuged at 3000rpm for 3 minutes. The supernatant was subsequently aspirated by pipette and discarded. The PBLs were resuspended in red cell lysis buffer, centrifuged and the supernatant discarded and then stained as described in section 2.2.3.2.

2.2.1.4. Cell isolation from heart tissues

Briefly, hearts were excised and rinsed with HEPES buffered DMEM (GIBCO 42430-082) to remove any blood clots and remaining blood. Tissue was cut into 2 mm pieces using a sterile scalpel, then transferred into 5ml bijoux and incubated with collagenase digestion mix (section 2.2.8) and incubated for 30 min in a 37^0C water bath. The suspension was then passed through a 70µm sieve (BD Falcon) to remove undigested tissue and returned to the water bath for a further 15 min. Collagenase action was stopped by adding ice cold working medium (RPMI, 10%FCS, 2ME, L-Glut, Streptomycin and Penicillin) and suspension was then washed x2 with DMEM. To ensure a final single cell suspension, the cells were incubated with Trypsin-EDTA (Sigma G1393) for 10 min at 37^0C and used as per experiment requirement.

2.2.1.5. $CD4^+$ T cell purification

Splenocytes were resuspended in 500µl of MACS buffer and passed through the autoMACS machine using program Possel and the negative fraction was collected; this procedure was effective at removing dead cells. The negative fraction was centrifuged and resuspended with 750µl of MACS buffer and 100µl of MHC class II beads (to remove the MHC class II positive cells). Cells were then incubated for 20 minutes on ice. After washing and resuspension in 500µl of MACS buffer the cells were passed through the autoMACS machine using the Possel program and the negative fraction was collected; this was centrifuged, resuspended with 750µl of MACS buffer and 100µl of CD4 beads, and incubated for 20minutes on ice. After washing and resuspension of cells in 500µl of MACS buffer, the cells were passed through the auto MACS machine using the possel-d program and the positive fraction was collected. Purified $CD4^+$ T cells were washed, counted, made up to the required volume in normal saline or Hartman's solution and injected into the animals according to the protocol of the experiment. This resulted in >97% purification of CD4 T cells.

2.2.2. Serum preparation

Approximately 20µL of blood was collected from the superficial tail vein of animals as described above and stored overnight at 4^0C. Samples were centrifuged twice at 13000rpm for 7 minutes, the serum was collected and subsequently heat inactivated at 56^0C for 30 minutes. Samples were stored at -20^0C until analysis.

2.2.3. Flow cytometry

2.2.3.1. Introduction and basic principle

Flow cytometry detects fluorescent-labelled antibodies or ligands that can be made to bind specific cell-associated molecules.

In my work, flow cytometry was used primarily for characterising cell surface expression molecules using fluorochrome-conjugated antibodies (immunophenotyping). This included

A) Characterisation of individual lymphocyte populations (like CD4 T cells, CD8 T cells and B cells) with special interest in characterisation of CD4 T cell subset like T_{FH} cells.
B) Phenotyping of animals
C) Characterisation of cell populations following in vitro cultures
D) To trucount the lymphocytes

This is based on the basic principle of single cell preparations successive binding steps of incubation with labelled antibodies or ligands. Each step was followed by wash with excess buffer to remove unbound reagents.

In multiple colour flow cytometry, I made various adjustments and controls in order to create optimal settings that offer adequate sensitivity but also minimise the background fluorescence and spectral overlap between different fluorochromes. Some protocols and control that were consistently used in my experiments are described below.

Fc Receptor (FcR) blocking

Several cell types express FcϒII (CD32) and FcϒIII (CD16) receptors for complexed IgG (Fc receptors or FcR), including NK cells, monocytes ad macrophages. Antibody preparations can bind to FcR-bearing cells through their Fc portions, regardless of their antigen specificity. To minimise background staining from FcR ligation, FcϒR sites were blocked by incubation with anti-CD16/CD32 antibodies prior to staining.

Dead cell exclusion

Cell membrane of nonviable cells can trap fluorescent reagents and therefore, dead cells were excluded from the analysis using the dead cell marker 7-AAD.

Autofluorescence control

I used unstained cells to establish levels of background autofluorescence, and to adjust voltage settings such that the unstained population of interest appeared in the first quartile of a 4-decade logrithmatic scale for each fluorochrome to be measured.

Single stain controls

The emission spectra of some fluorochrome can overlap causing fluorescence from one fluorochrome to spill into the other. To overcome this problem, control cells were stained with single fluorochromes separately and were thus used a compensation controls to calculate any spectral overlap which was accounted in data analysis.

2.2.3.2 General protocol

Flow cytometry was carried out using single cell suspension of lymphocytes in 96-well U-bottomed plates (BD, Franklin Lakes, NJ, USA). Cells were washed (with 150 µl/well of FACS buffer here and hence forth), blocked with 50µg/ml anti-mouse CD16/CD32 (clone 2.4G2, BD Pharmingen, San Diego, CA, USA) at 5µl/ml in FACS buffer for 30 minutes at 4°C. Cells were then incubated with dead cell exclusion dye 7-AAD (BD Pharmingen, San Diego, CA) and/or the indicated primary and subsequently secondary monoclonal antibodies in different dilutions (mAb) (table 2.3.) in the dark for 30 min at 4°. Biotinylated antibodies were detected with allophycocyanin-conjugated streptavidin (BD Pharmingen, San Diego, CA) or APC-Cy 7 conjugated to streptavidin (BD Pharmingen). Prior to analysis cells were transferred to flow cytometry tubes (BD Falcon, Franklin Lakes, NJ, USA) and cells analysed on a FACSCanto II flow cytometer with FACSDiva (BD Biosciences, San Jose, CA) and or on FlowJo (Tree Star, Inc, Ashland, OR) software.

2.2.3.3. Trucount™ analysis for cell quantification

When required, a 20µl aliquot of the single cell suspension was stained to label the populations of interest and then added to a BDTrucount™ tube (BD Biosciences) containing a known number of fluorescent beads. It was mixed thoroughly and run through the flow cytometer. The number of events in the selected gate was compared to the number of beads in the same run to calculate the density of the cell population of interest in the sample.

Table 2.3. Antibodies used for flow cytometry

Antigen specificity/ (Clone)	Conjugate or fluorochrome	Dilution	Isotype/ (Source)
Mouse CD4 GK1.5	Biotin	1:500	Rat IgG2b, k BD Pharmingen™
Mouse CD4 L3T4	PeCy7	1:1000	Rat IgG2b, k BD Pharmingen™
Mouse CD4 RM4-5	APC	1:500	Rat IgG2b, k BD Pharmingen™
Mouse CD11c HL3	PE	1:50	Rat IgG2b, k BD Pharmingen™
Mouse CD19 ID3	PE	1:200	Rat IgG2a BD Pharmingen™
Mouse CD19 MB19-1	FITC	1:200	Rat IgG2a, k BD Pharmingen™
Mouse CD44 IM7	FITC	1:100	Rat IgG2b, k BD Pharmingen™
Mouse CD62L MEL-14	PeCy7	1:1000	Rat IgG2a, k BD Pharmingen™
Mouse CXCR5 2G8	Biotin	1:100	Rat IgG2a, k BD Pharmingen™
Mouse CXCR5 2G8	APC	1:100	Rat IgG2a, k BD Pharmingen™
Mouse CXCR5 2GB	Purified	1:100	Rat IgG2a, k BD Pharmingen™
Mouse PD-1 (CD297) J43	PE	1:500	Armenian Hamster IgG_2, k E Biosciences
Mouse ICOS 7E.17G9	PE	1:500	Rat IgG2b, k BD Pharmingen™
Rat Thy1.1	APC-Cy7	1:1000	Mouse IgG

OX-7			BD Pharmingen™
7-AAD	Percp-cy5.5	1/200	BD Pharmingen™
GL7 (GL-7)	Biotin/FITC	1:100	Rat IgM, Affymetrix UK
Mouse CD45.1 A20	APC/Cy7	1/100	Rat IgG2a, k BD Pharmingen™
Mouse CD45.1 A20	Biotin	1/500	Rat IgG2a, k BD Pharmingen™
Mouse CD45.2 104	APC/Cy7	1/100	Rat IgG2a, k BD Pharmingen™
Mouse CD45.2 104	Biotin	1/500	Rat IgG2a, k BD Pharmingen™
Mouse CD105	APC	1/100	Rat IgG2a, k
Mouse Isoselectinβ4	APC	1/100	Rat IgG2a, k

2.2.4. Indirect immunofluorescence Hep-2 analysis

2.2.4.1. Background and basic principle

Indirect immunofluorescence hep-2 analysis was used for measurement of autoantibody. It is an excellent screening test for detection of autoimmune disorders but not specific. Our laboratory adapted this screening test to study the development of autoantibodies following solid organ transplantation.

In the indirect immunofluorescence technique, samples are incubated with antigen substrate and unreacted antibodies are washed off. Then the substrate is incubated with specific fluorescein labelled conjugate and unbound reagent is washed off. On microscopy, autoantibody positive samples exhibit an apple green fluorescence corresponding to areas of the cells or nuclei where autoantibody has bound.

2.2.4.2. Protocol

The presence of antinuclear autoantibodies (ANAs) was determined by HEp-2 indirect immunofluorescence. Test sera were diluted 1:10 in PBS and incubated for 30 minutes on slides coated with HEp-2 cells (from Hep-2 ANA – IFA Kit; Binding Site, Birmingham, UK). ANAs were then detected with FITC-conjugated anti-mouse IgG mAb (STAR 70; Serotec, Oxford, UK). Serial two-fold dilutions (1 in 10 to 1 in 2560) of hyperimmune sera from *TCR*$^{bd-/-}$ animals injected iv with 5x10^{6} purified bm12 CD4 T cells, was used to generate a standard curve (figure 2.1). Monospecific positive and negative prediluted controls (Hep-2 ANA – IFA Kit as above) were used according to the manufacturer's instruction. Pooled sera from naïve BL6 animals were used as an additional negative control.

2.2.4.3. Analysis of slides

For each test serum, 5 photomicrographs were taken and the intensity of staining determined by integrated morphometric analysis using MetaMorph software or image J software. The relative fluorescence score (fluorescein binding units) was then derived by comparison to a standard curve of hyperimmune serum that was assigned an arbitrary value of 1000 fluorescence binding units.

2.2.5. ELISA

2.2.5.1. Background and basic principle

ELISA is an immunoassay for the detection of an antibody or an antigen in a test solution. The assay was developed as an alternative to the radio immunoassay which posed a potential health threat.

I used this assay to detect and quantify the development of alloantibody and autoantibody in serum samples following a challenge.

2.2.5.2. Protocol for detection of alloantibody

96-well ELISA plates were (Immulon 4HBX, Thermo, Milford, MA) with antigen diluted in Na_2CO_3-$NaHCO_3$ buffer [pH 9.6]) and incubated overnight at 4°C. Non-specific binding sites were blocked with Marvel dried skimmed milk powder (Premier International Foods, UK) in PBS (block) at 200 µl/well for 2 hours at 37°C. After washing (x6 with PBS/0.05% tween 20 [Sigma, Poole, UK] in PBS throughout), samples were diluted 1:9 in block and 50 µl/well added. Serial tripling dilutions were carried out down the 8 rows of the plate, and the plate incubated at 37°C for one hour. Bound IgG antibodies were detected by incubating for 1 hour at 37°C with biotinylated rabbit $F(ab')_2$ anti-mouse IgG (1/1000 in block; STAR11B; AbD Serotec, Oxford, U.K.) and Extravidin Peroxidase conjugate (1/1000 in block; Sigma, Poole, U.K.). Before addition of secondary antibody and between all subsequent steps, plates were flicked and washed 6 times with PBS/0.05% tween. In order to generate a colorimetric signal, Sure Blue substrate (KPL, Gaithersburg, MD) was added to each well and reaction was stopped by the addition of 0.2 M H_2SO_4. Plates were read in the FLUOstar OPTIMA plate reader (BMG Labtech, Aylesbury, U.K.) at 450nm.

Analysis of results

For each sample, an absorbance versus dilution curve was plotted and the area under the curve calculated (Callaghan et al., 2007). The AUC was then calculated as a percentage of the AUC of a standard of serial diluted pooled hyperimmune sera (sera pooled from BL6 animals transplanted with BALB/c skin or cardiac grafts collected at week 5 post-transplant – that was assigned an arbitrary value of 100%. Pooled sera from naïve BL6 or $TCR^{bd-/-}$ animals were used as a negative control. The capturing antibodies and incubations specific to the different ELISAs based on this method are summarized in table 2.4.

Table 2.4. Protocol for alloantibody ELISAs

Analyte	Coating agent	Positive control	Development time (seconds)
Anti-H2-K^d antibody	Recombinant conformational H2-K^d at 5µg/ml	H2-K^d immune serum	90

2.2.5.3. Protocol for detection of anti-vimentin autoantibody

Anti-vimentin antibody was detected by performing anti-vimentin antibody ELISA which is adopted by modifying the protocol as described by Mahesh et al (Mahesh et al., 2007). Mouse vimentin was plated at 1 µg/well onto 96-well ELISA plates and incubated overnight at 4°C. Nonspecific binding sites on the plates were blocked with phosphate-buffered saline (PBS) with 0.1% Tween 20 and 5% milk (block) for 2 hours. For each ELISA, standard curve was plotted using doubling dilutions of murine monoclonal IgG anti-vimentin antibody which cross-reacts with murine vimentin (clone RV202, 500 µg/ml; BD Biosciences, Oxford, UK) beginning at 1:4000 dilution in block. Experimental samples (50 µl, diluted 1/100 in block) were incubated for 1 hour at 37°C followed by wash by PBS/0.05% tween six times. Bound IgG anti-vimentin Abs were detected by incubating for 1 hour at 37°C with biotinylated rabbit $F(ab')_2$ anti-mouse IgG (1/1000 in block; STAR11B; AbD Serotec, Oxford, U.K.) and ExtrAvidin Peroxidase conjugate (1/1000 in block; Sigma, Poole, U.K.). Before addition of secondary Ab and between all subsequent steps, plates were flicked and washed 6 times with PBS/0.05% TWEEN. Sure Blue substrate (KPL, Gaithersburg, MD) was then added, the reaction was stopped at 10 minutes by the addition of 0.2 M H_2SO_4, and the absorption at 450 nm was measured in a FLUOstar OPTIMA plate reader (BMG Labtech, Aylesbury, U.K.). Standards were used to determine the titre of the samples. For each ELISA a standard curve was plotted, and the OD readings were read off the standard curves to obtain relevant IgG anti-vimentin titres.

2.2.6. ELISPOT

2.2.6.1. Background and basic principle

ELISPOT is highly sensitive assay that detects ASCs or cytokine secreting cells at a cell level ex vivo. This was developed as a stable alternative to the conventional plaque forming cell assays that relied on haemolytic lysis of Protein A coated sheep red blood for the detection of ASCs.

In my work, I used this assay to quantify the antigen-specific IgG and dsDNA antibody secreting cells (ASCs) in the spleen and bone marrow.

Like ELISA, following steps were taken to avoid any non-specific binding of the antigen and antibody.

- High affinity antibodies that displayed negligible cross-reactivity were used for capture and detection.
- Non-specific binding was minimized by incubating coated plates with serum containing blocking solution to saturate available sites prior to addition of cells.
- Relevant positive and negative biological controls were also included in each test run.
- Unbound reagents were removed after each step and the plates were washed with mild detergent solution to minimize non-specific binding.

2.2.6.2. General Protocol

96-well MultiScreen Filter Plates were coated (Millipore, Billerica, MA, USA) with 100µl/well of antigen diluted sterile buffer. The plates were incubated for 2 hours at 37°C, 5% CO_2 followed by 5 times wash with sterile PBS + 0.5% BSA (hereafter referred to as 'PBS buffer). Then 100µl/well full media (RPMI culture medium + 10% FCS, 1% penicillin-streptomycin [Sigma, Poole, UK] + 1% L-Glutamine 200mM [Sigma] and + 0.1% 50mM 2mercaptoethanol [2ME; Sigma]) was added. Single cell suspensions from spleen and BM were prepared by pushing through a 40µm falcon cell strainer and resuspended at 1 x 10^6/ml in full media and added to the plate in triplicates of 1x10^5, 1x10^4 and 1x10^3 cells per well. The plates were incubated for 18 hours at 37°C, 5% CO_2, ensuring the plate was not moved within this time.

After washing (x10) with 0.05% tween in PBS (hereafter referred to as 'wash buffer') and patting dry, 100µl/well biotinylated Rabbit(ab')2 anti-mouse IgG (STAR11B, AbD Serotec, Oxford, UK) at 1µl/ml in in PBS buffer for 2 hours at 37°C. After washing (x6) with wash buffer, the plate was incubated with 100µl/well ExtrAvidin Peroxidase conjugate (Sigma, Poole, UK) 1µl/ml in PBS buffer for 2 hours at RT.

To make the developing solution, one tablet of 3-amino-9-ethyl-carbazole (AEC; Sigma, Poole, UK) was added to 2.5ml dimethyl formamide (DMF; Sigma, Poole, UK), 1 ml of the resulting solution was added to 19 ml acetate buffer, filtered through a 0.2µm filter (Sartorius Stedim, Surry, UK) and 10µl of 30% Hydrogen peroxide solution (Sigma, Poole, UK) added. After washing (x6), spots were developed by incubation with 100µl/well of the above developing solution in the dark at RT till spots appeared. The plate was washed (x3) with distilled water and left overnight in the dark to dry before being read on an AID^{TM} Elispot Reader version 3.5 (Autoimmun Dignostika, Strasberg, Germany). Data was expressed as mean number of responders per 10^6 cells (± SD).

The coating agents, dilutions and development times for specific to the different ELISPOT assays based on this method have been summarized in table 2.5.

Table 2.5. Summary of various ELISPOT assays

Specificity	Capturing coating agent	Concentrations and diluting buffer	Development time (min.)	Pre-treatment of plates
Pan IgG	Rabbit anti-mouse IgG (STAR 8B; Serotec, Oxford UK)	2µg/ml diluted in 0.1M bicarbonate buffer (Na_2CO_3-$NaHCO_3$, pH 9.6).	15	NIL
Anti-dsDNA antibody	dsDNA (produced in house)	20µg/ml in sterile distilled water	30	Pre-coat plates with 20µg/ml poly-L-Lysine

2.2.7. Histology

2.2.7.1. Background and basic principles

Histology reveals the relationship between the different types of cells and tissue types found within tissues and organs in health and disease. Although it is less quantitative than other cellular and biochemical assays, it allows visual analysis of localized tissue differences, thus giving greater insight into the cellular and molecular interactions that may be significant for disease progression.

I used tissue histology to assess

A) Donor heart grafts for the development of allograft vasculopathy and C4d deposition by immunohistochemistry.
B) Recipient spleens for the development of germinal centres and T-B cell interactions by immunofluorescence.

2.2.7.2. Protocol for tissue preparation

General histology

Tissues were placed in 10% formalin saline solution and paraffin-mounted by the Department of Pathology, Papworth Hospital, Cambridge, UK.

Immunohistochemistry and immunofluorescence

Tissues were embedded in OCT compound (VWR International, USA), flash frozen in liquid nitrogen, and stored at -80 °C. Frozen tissues were cut into 7µm serial sectioned and placed onto Poly-L-Lysine coated slides (Sigma Aldrich Inc.) After drying for 30minutes, the tissue sections were fixed in chilled acetone for 10minutes. Sections were then air dried for 30 minutes and stored at -80°C.

2.2.7.3. Protocol for tissue staining

General histology

Paraffin fixed sections were stained by the Department of Pathology, Papworth Hospital, Cambridge, UK with either Haematoxylin and Eosin (H&E) or elastin van Gieson (EVG) for the assessment of arterial vasculopathy and parenchymal rejection of transplanted hearts.

Immunohistochemistry staining

C4d staining was performed under supervision of Sylvia Rehakova. 7µm sections of tissues were stained by using the Avidin- Biotin- Peroxidase complex (ABC) technique (Vector Laboratories Inc.) for detection of the complement by-product C4d. Sections were thawed and rehydrated with 1%PBS, and tissue peroxidase activity was quenched by incubation with 0.3% H_2O_2 in PBS for 10 minutes. The sections were washed in PBS and then endogenous biotin sites on the tissues was blocked by using Avidin and Biotin blocking solution, each for 15min. Non-specific binding sites were blocked by incubating sections with 5% Rabbit serum (Sigma) in PBS for 20min. Sections were incubated with primary antibody (table 2.6) diluted in PBS for 30min at RT. The bound primary antibody was detected by incubation with biotinylated secondary antibody followed by incubation with ABC solution [avidin and biotinylated horseradish peroxidase (HRP) complex] solution (Vector Laboratories Inc.) each for 30min. All incubations were carried out in humidified chamber followed by two 5min washes in PBS. For development, sections were incubated with 0.3mg/ml DAB with 0.03% hydrogen peroxide for 30 seconds. The sections were subsequently counterstained with Harris' haematoxylin (Sigma Aldrich Inc.) for one second. Sections were dehydrated with increasing concentration of ethanol, cleared in xylene and mounted in DPX (Sigma Aldrich Inc.).

Immunofluorescence staining

Non-specific binding sites were blocked by incubating sections with 5% BSA (Sigma Aldrich Inc.) and 4% goat anti mouse serum (Sigma Aldrich Inc.) for one hour at RT. Endogenous biotin was blocked by incubating tissue section with avidin and biotin blocking kit solutions (Vector Laboratories Inc.), each for 15 min and washed three times each for 10 minutes with 1%PBS after every step. Sections were then incubated with primary antibodies conjugated to a fluorochrome; or antibodies conjugated to biotin, followed by incubation with fluorochrome conjugated secondary antibody (Table 2.6). Antibodies were diluted in blocking BSA-goat anti-mouse serum and incubated for one hour in dark humidified chamber followed by three washes in excess PBS for 10min. However, in case of confocal microscopy, primary antibodies were incubated overnight at 4°C in dark humidified chamber.

Sections were counterstained with 20% Harris' haematoxylin (Sigma Aldrich Inc.) for 20 seconds, washed in excess water and mounted in Fluor Save^{TM} reagent (Calbiochem^{R}, Merck-Millipore). Where required, sections were counterstained and mounted with vectashield's hard set mounting medium with 4' , 6- diamidino-2-phenylindole (DAPI)) (Vector Laboratories Inc).

Anti-mouse antibodies which were used for immunofluorescence staining of splenic sections are shown in table 2.6.

Table 2.6. Antibodies used for immunohistochemical staining

Primary antibodies			Secondary antibodies	
Antigen specificity/ (Clone) (dilution)	**Conjugate or fluorochrome**	**Isotype/ (Source)**	**Clone (dilutions)**	**Source**
Mouse B220 (RA3-6B2) (1/200)	Purified	Rat IgG2b kappa (BD Pharmingen ™)	Cy3 polyclonal goat anti rat IgG (1/500 dilution)	Jackson Labs
Mouse B220 (RA3-6B2) (1:200)	APC	Rat IgG2b kappa (BD Pharmingen ™)	NIL	
Mouse GL7 (GL-7) (1/100)	FITC	Rat IgM (Affymetrix UK Ltd.)	NIL	
Mouse GL7 (GL-7) (1/100)	Biotin	Rat IgM (Affymetrix UK Ltd.)	Cy2 streptavidin Polyclonal goat anti rat IgG (1/500 dilution)	Jackson Labs
PNA (1/200)	Fluorescein	Vector laboratories Inc.	NIL	
Mouse C4d (16D2) (1/100)	Purified	Abcam® , UK	Biotinylated polyclonal rabbit anti rat IgG (1/500)	Abcam® , UK
Mouse CD4 GK1.5 (1/200)	Biotin	Rat IgG2b kappa BD Pharmingen™	Streptavidin Alexa Fluor 555 (1/500)	Invitrogen
FDC FDC-M1 (1/250)	Purified	Rat IgG 2c	Cy3 polyclonal goat anti rat IgG (1/500)	Jackson Labs

2.2.7.4. Imaging

I used conventional and confocal microscopy to analyze tissue sections. Conventional microscopy was used for quantification of GCs in recipient spleens and calculation of AV in donor heart grafts. However, confocal microscopy was used to study T and B cell interactions in recipient splenic sections following transplant or cell challenge.

In confocal microscope, presence of confocal pinholes removes out of focus light from the image thus generating images of improved resolution. It has a higher level of sensitivity compared to conventional microscope due to highly sensitive light detectors and its ability to accumulate the captured image over time.

Conventional Microscope

Sections were viewed using an IX81 microscope at 20x magnification, 70 UplanApo Lense (Olympus, Japan). Images were photographed using an ORCA-ER digital camera (Hamamatsu Photonics, Japan) and acquired with CellR 2.6 software (Olymous Imaging Solutions, Germany).

Confocal Microscope

For confocal microscopy, sections were viewed using a Zeiss Laser Scanning Microscope 700 (LSM 700) (Carl Zeiss AG). Images were photographed using ZEN 2011 (Carl Zeiss AG) imaging software.

2.2.7.5. Analysis of results

Histological evaluation of allograft vasculopathy and parenchymal rejection

Allograft vasculopathy was assessed on elastin van Gieson-stained paraffin sections by morphometric analysis, using digital imaging software (Cell^R, Olympus, Japan) to calculate luminal stenosis as follows.

$$\text{Percentage cross-sectional area luminal stenosis} = \frac{\text{Area within internal elastic lamina (IEL)} - \text{Area of lumen}}{\text{Area within IEL}} \times 100$$

All elastin-positive vessels in each section were evaluated, with an average of 10 vessels per heart examined.

Quantification of germinal centres

The development of germinal centres within the secondary lymphoid organs in the recipients of allograft was quantified by staining with B220 and PNA or B220 and GL7. The percentage of follicles with GCs was derived by dividing the PNA or GL 7 positive follicles to total number of B220 positive follicles within a good recipient splenic section.

$$\text{Percentage of GCs} = \frac{\text{Number of PNA}^{+} \text{ or GL7}^{+} \text{ GCs}}{\text{Total number of B220}^{+} \text{ follicles}} \times 100$$

2.2.8. Endothelial migration assay

2.2.8.1. Background

Cell migration of quiescent cells in response to biological insults can indicate cell activation. Thus migration may be the result of a physiological response to insult such as wound healing, but may sometimes contribute to disease pathogenesis. Activation of ECs by antibody and complement is thought a contributing factor to the development of AV. Cell invasion assays allow in vitro quantification of cell activation in response to specific insults.

In my work, I used migration of ECs as an in vitro test for EC activation following exposure to antinuclear autoantibodies.

2.2.8.2. Procurement of tissues and Preparation

Hearts were harvested from two weeks old new born babies to get highest yield of endothelial cells (ECs). In our experience, we get good yield of ECs if procured from new born babies and are more than 8 in numbers. Mouse endothelial cells were cultured using a previously described protocol. Briefly, single cell suspension from hearts was achieved by following the protocol as mentioned in 2.2.1.4.

2.2.8.3. MACS purification endothelial cell

The resultant cell suspension was washed once with working medium, cell button was resuspended in 500µL of MACS buffer (Mitenyi Biotec 130-091-221) and incubated with Fc gamma receptor blocking antibody (BD rat anti-mouse CD16/CD32) 1/200 dilution for 30 min on ice. Biotinylated antibodies were added to the suspension: CD31, 1/100 (BD 553371); CD105, 1/200 (eBioscience 13-1051) and isolectin B4 (Vector B-1205), 1/25 dilution in MACS buffer and incubated for 30 min on ice. CD31 (PECAM-1), CD105 (endoglin) and isolectin B4 are all expressed on EC surface. Suspension was washed in MACS buffer, 50µL of anti-biotin beads were added (Miltenyi Biotec 130-090-485) and incubated for 15min at 4^0C. Cells were washed in MACS buffer as before. Cell separation was done using autoMACS Column machine (Miltenyi Biotec) using Possel program and collecting the positive fraction.

2.2.8.4. Endothelial Cell Culture

Purified endothelial cells seeded in 1% gelatine (Sigma) coated flasks with 5 ml of Growth Medium and incubated at 37^0C with 5% CO_2. 24 hours later all non-adherent cells were removed using a sterile pipette and 10-15 ml of fresh growth medium added. Medium was

changed every 72 hours and cells left to grow until reaching ~80-90% confluence at which point cells were passaged, using Cell Dissociation Solution (Sigma C-5789). Endothelial cells for experiments were used at passage 3-6; preferably just after 3.

Collagenase Digestion Mix

1 ml Collagenase (1mg/ml) (Roche 10103586001)

20µL DNAse stock (1mg/ml) (Roche 1284932)

200µl FCS (sigma 4135)

8.8ml HEPES buffered DMEM (GIBCO 42430-082)

Growth Medium (50ml)

37.5 mls HEPES buffered DMEM (GIBCO 42430-082)

10 mls FCS (Sigma 4135)

50µL (50 mM) of 2ME (Sigma 516732)

500µL P/S (Sigma 4333)

500 µL L/Glut (Sigma 6392)

500 µL MEM non-essential amino-acids (Sigma M7145)

500 µL Sodium Pyruvate (Sigma S8636)

500 µL Endothelial Cell Growth Factor (Sigma E9640)

2.2.8.5. Endothelial migration assay

In vitro wound induced endothelial cell migration was assessed using a previously described protocol (Herve et al., 2005). ECs were seeded in 8cm Petri dishes, pre-coated with 1% gelatine, and were incubated in full endothelial growth medium(see above) until confluence. Cells were then "starved" for 24hrs, growth medium was then aspirated and instead cells were incubated with Minimal medium (1% FCS, 1% penicillin-streptomycin, 2mM L-Glutamine, 0.05 mM 2ME, 1mM Sodium Pyruvate, MEM Non-essential amino acids) that lacked any endothelial cell growth factor for 24h to minimize baseline proliferation. A rectangular lesion was made in the monolayer using a sterile (1000µl) pipette tip across the

diameter of the dish. Cells were rinsed with PBS and then incubated with serum of interest, 1:500 dilution, for a further 24 hrs. Cells were washed with PBS to remove serum and fixed with paraformaldehyde (BD Cytofix kit 554714) for 30 min. Cells were then stained with Crystal Violet 0.05%. Dishes were then washed x2 and allowed to dry. The number of cells which had moved across the starting line was measured. Five fields were analysed for each well at 4x magnification and expressed as mean ± SD.

Furthermore, in order to remove any confounding factors to this assay because of the presence of complement factors and other proteins in serum; autoantibody was column-purified using a column purification kit as per manufacturer instructions (NAb™ Protein G Spin Purification Kit, Pierce, Rockford, IL, USA). Cells were incubated with immunoglobulin from serum of transplanted mice, or with antibodies of interest for a further 24-36h, fixed with paraformaldehyde (BD Cytofix kit, BD Biosciences), and then stained with 0.05% Crystal Violet solution. The negative fraction from column purification was used as negative control. For positive control, commercial anti-H-2D^{b} mAb (BD Pharmingen, San Diego, CA, USA) was added to endothelial cells (diluted in medium in 1/200). For each plate, five fields along the lesion were analysed and numbers of cells encroaching the lesion were counted using light microscopy.

2.2.9. Polymerase chain reaction

2.2.9.1. Background and basic principle

The polymerase chain reaction (PCR) allows the in vitro amplification of selected regions of DNA. This assay is relatively rapid and highly sensitive, often requiring only small amounts of template DNA for amplification.

In my work, I used this assay

- To genotype genetically modified (GM) mice
- And to selectively amplify cDNA coding for transcriptional factor B cells lymphoma factor 6 (Bcl-6) for T_{FH} cells

The PCR entails repeated cycles of heating and cooling of the reaction mixture for the enzymatic replication of DNA. The reaction mixture contains template DNA, single standard DNA oligonucleotides called primers that contain amino acid sequences complementary to the start and end of the target region to be amplified, free nucleotides, and the enzyme DNA polymerase suspended in an appropriate buffer.

Firstly, high temperatures are used to denature the DNA helix into two linear strands. Then mixture is cooled to facilitate association of primers to the single stranded DNA. The temperature is then adjusted to help enzymatic extension of the bound primer by DNA polymerase, for generation of a strand complementary to the template DNA, using free nucleotides in the reaction mixture. The series of steps is repeated and replicated, fragments from each cycle act as template in subsequent cycles, allowing exponential accumulation of target DNA. Heat-stable DNA polymerases that can tolerate successive cycles of high temperatures during denaturation allow the reaction to proceed uninterrupted in a thermal cycler.

The 5’ ends of the primers can be modified to incorporate new nucleotide sequences at either end of the selected DNA region; this was used in my work to introduce terminal restriction endonuclease sites, in order to facilitate subsequent cloning of the amplified fragments into mammalian expression vectors.

2.2.9.2. Protocol

Primer design

Primers were designed using commercial software provided by Sigma Aldrich Inc. (Table 2.7). When required, restriction endonuclease recognition sites were added at the 5' ends of primers for downstream cloning of amplified DNA into expression vectors. A detailed sequence of primers used is shown in table 2.8 and 2.9. All primers were ordered from Sigma Aldrich Inc.

PCR conditions

In case of genotyping the following mixture was used.

- 10mM dNTP (Sigma Aldrich Inc)-1.25µl
- 10µM forward primer- 2µl
- 10µM reverse primer-2µl
- 2.5mM MgCl2 (Applied Biosystems, Life Technologies Inc.)- 2.5µl
- 1µg of plasmid DNA
- 1 unit Amplitaq Gold DNA Polymerase- 1.25µl
- Betaine- 5µl
- PCR 10x buffer in a 40µl volume-2.5µl

The details of various primers and protocol are given in table 2.9.

In case of amplification of DNA for Bcl-6 viral transduction, I used phusion high-fidelity DNA polymerase (New England BioLabs).

- 10mM dNTP (Sigma Aldrich Inc)-0.5µl
- 10µM forward primer-1.25µl
- 10µM reverse primer-1.25µl
- 5x phusion buffer-5µl
- 1µg of plasmid DNA-5µl
- 0.25µl phusion DNA Polymerase-0.25µl
- Water to ,make it to 25µl

PCR amplifications were performed in Biometra T3 Thermocycler (Biometra, Goettingen, Germany).

Analysis of PCR products

PCR products were run in 1% Agarose gel as described below in section 2.2.10 for analysis and further purification.

Table 2.7 Parameters for primers

Parameters	
Primer length	15-25 bases
Melting temperature (Tm)	55-65 ^{0}C
Tm difference between forward and reverse primers	<5^{0}C
Annealing temperature (Ta)	10^{0}C lower than the lowest Tm of the primers
Percentage GC content	40-60%
Secondary structure and self-complimentary	Selected the ones which did not possess secondary structures

Table 2.8. Primers used in cloning

Primer	**Sequence**
For lentiviral cloning of Bcl-6	
$BCL6_{for}$	ATGGCCTCCCCG GCTG
$BCL6_{rev}$	TCAGCAGGCTTTGGGGAG
$BAMHIBCL6_{for}$	CGTACAGGATCC ACG ATG GCCTCCCCGGCTG
$BAMHIBCL6_{rev}$	CGTACAGGATCCACTTCAGCAGGCTTTGGGGAG
BCL6 $middle_{for}$	CATCGTTCTCAACAGCCTCA
BCL6 $middle_{rev}$	TGAGGCTGTTGAGAACGATG
$BCL6_{rev156position}$	AAGACAGTGCTCATGGCC

Table 2.9. Genotyping of animals

Animal	Primers	Thermocycler protocol
SAP$^{-/-}$.BL6 On gel electrophoresis, band should be present at 350bp with absent WT band at 1000bp	(Fwd) GACATCTTATCCTCCAGTTTTATT (rev) AAAGTTTGTAAAGACACTATAAGTTA	Denaturation 94^{0}C-2min 94^{0}C-30sec x 35 cycles Annealing temperature 60^{0}C-40sec x 35 cycles 72^{0}C-2min x 35 cycles Final extension 72^{0}C-2min
TCR$^{bd-/-}$ On gel electrophoresis, Beta and delta knockout bands should be present at 280bp with absent WT bands.	(β WT fwd) TGT CTG AAG GGC AAT GAC TG (βWT rev) GCT GAT CCG TGG CAT CTA TT (δ WT fwd) CAA ATG TTG CTT GTC TGG TG (δ wt rev) GTC AGT CGA GTG CAC AGT TT (KO fwd) CTT GGG TGG AGA GGC TAT TC (KO rev) AGG TGA GAT GAC AGG AGA TC	Reaction 1. For TCR beta gene Denaturation 95^{0}C-10min 95^{0}C-30sec x 35 cycles Annealing temperature 54^{0}C-40sec x 35 cycles 72^{0}C-1min x 35 cycles Final extension 72^{0}C-10min Reaction 1. For TCR delta gene Denaturation 95^{0}C-10min 95^{0}C-30sec x 35 cycles Annealing temperature 54^{0}C-40sec x 35 cycles 72^{0}C-1min x 35 cycles Final extension 72^{0}C-10min

Bm12 On gel electrophoresis, in bm12 reaction, for bm12 positivity, band should be present at 268bp with negative WT band. In BL6 reaction, band should be negative for homozygous bm12 status.	(Bm12 fwd) GAG TTC CTG GAG CAA AAG (bm12/BL6 rev shared) CTG CTG GGC CTC ATG AG (BL6 Fwd) GAG ATC CTG GAG CGA AC	For bm12 band Denaturation 95^0C-10min 95^0C-30sec x 35 cycles Annealing temperature 55^0C-30sec x 35 cycles 72^0C-1min x 35 cycles Final extension 72^0C-3min For BL6 Denaturation 95^0C-10min 95^0C-30sec x 35 cycles Annealing temperature 55^0C-30sec x 35 cycles 72^0C-1min x 35 cycles Final extension 72^0C-3min
DMa On gel electrophoresis, DMa KO band should be present at 520bp with a lack of BL6 band at 270bp.	(DMa WT) CAC ATT CCG GCA CAC TCT ATT CTG (DMa shared) TCT GGA CAC TGG GAT TTG ACC TTC (DMa KO) CCT TCT ATC GCC TTC TTG ACG	Denaturation 95^0C-10min 95^0C-30sec x 35 cycles Annealing temperature 60^0C-1min x 35 cycles 72^0C-1min x 35 cycles Final extension 72^0C-5min

2.2.10. Analysis of DNA

2.2.10.1. Analysis of DNA by Agarose gel electrophoresis

Background and principle

Gel electrophoresis is a laboratory method used to separate mixtures of DNA, RNA and proteins according to their molecular sizes. In gel electrophoresis, the molecules are pushed by electrical field through a gel that has small pores. The molecules travel through the gel pores at a speed that's inversely proportional to their sizes. It means that smaller molecules travel long distances than the larger molecules. It is fast and simple technique and with a sensitivity to detect as little as 1-10ng of nucleic acids. Bands can be readily recovered from the Agarose gel for downstream applications.

I used this technique to

- Measure the size and purity of DNA fragments following PCR and restriction endonuclease digestion
- To identify and isolate DNA fragments of interest

Protocol

Gels were cast in UV transparent plastic trays placed in a gel caster (Bio Rad Laboratories Inc.) powered Agarose was dissolved in Tris-acetate (TAE) buffer (pH8) by heating at low power in a microwave oven for 2-3minutes till a clear transparent solution was achieved. The solution was cooled to 60°C and supplemented with SYBR Safe DNA Gel Stain (Invitrogen, Life, technologies Inc.). The agarose solution was poured evenly onto the tray to a 1.0 cm depth approximately and gel was allowed to settle for 30-45 minutes at room temperature on a level bench. The tray was placed in the Sub-cell GT Cell (Bio Rad Laboratories Inc.) electrophoresis chamber filled with sufficient TAE buffer to submerge the gel.

Preparation of DNA samples

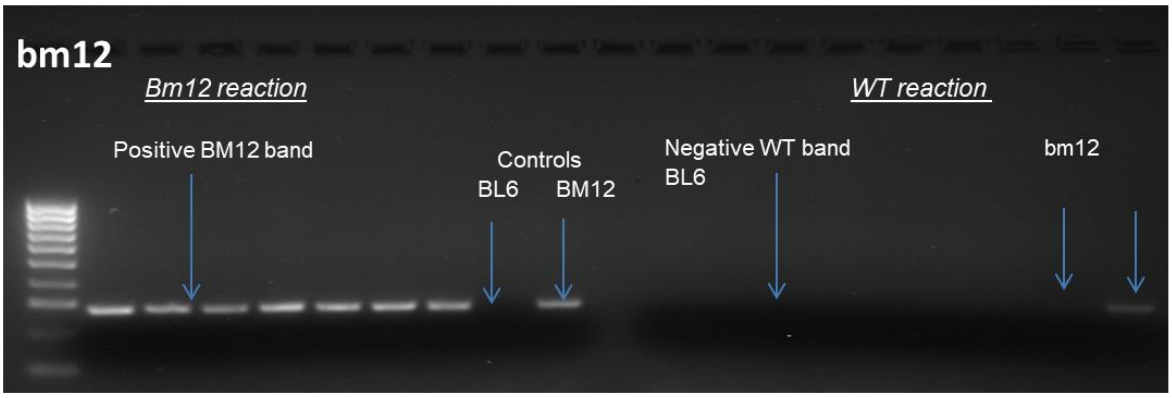
DNA samples were prepared by diluting them with a glycerol loading buffer (5% Glycerol, Bromophenol blue and xylene cyanol in 1M Tris). The bromophenol blue dye also moves to the anode at the rate of 300bp fragment, helping track loading and migration of sample.

Gel Run

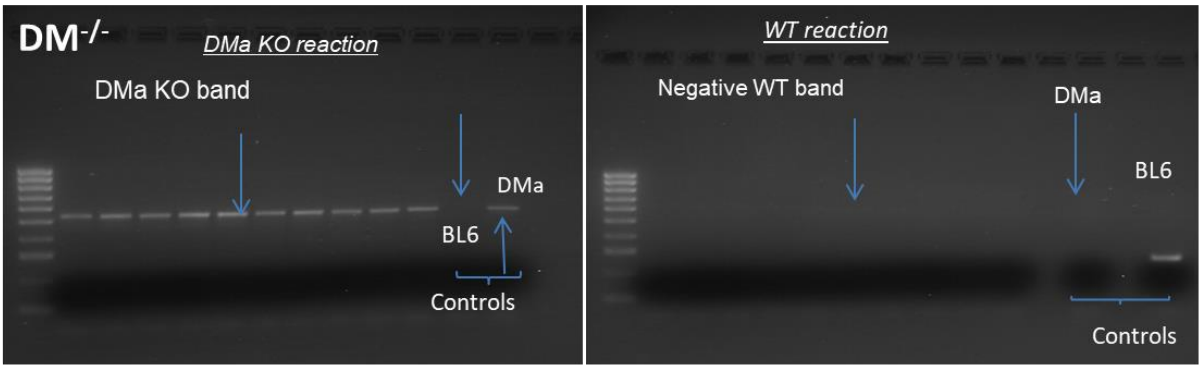
DNA samples were applied to the gel and a voltage of 100 V was applied across the electrophoresis tank for approximately 30 minutes or till the loading blue dye reached the bottom edge of the gel. Gels were visualised and photographed using the Gene Gnome Chemiluminescence Imaging System (Syngene UK, Cambridge, UK).

For genotyping, different bands of DNA for different animals and different reactions are shown below in figure

a. bm12



b. DM$^{-/-}$



c. SAP$^{-/-}$

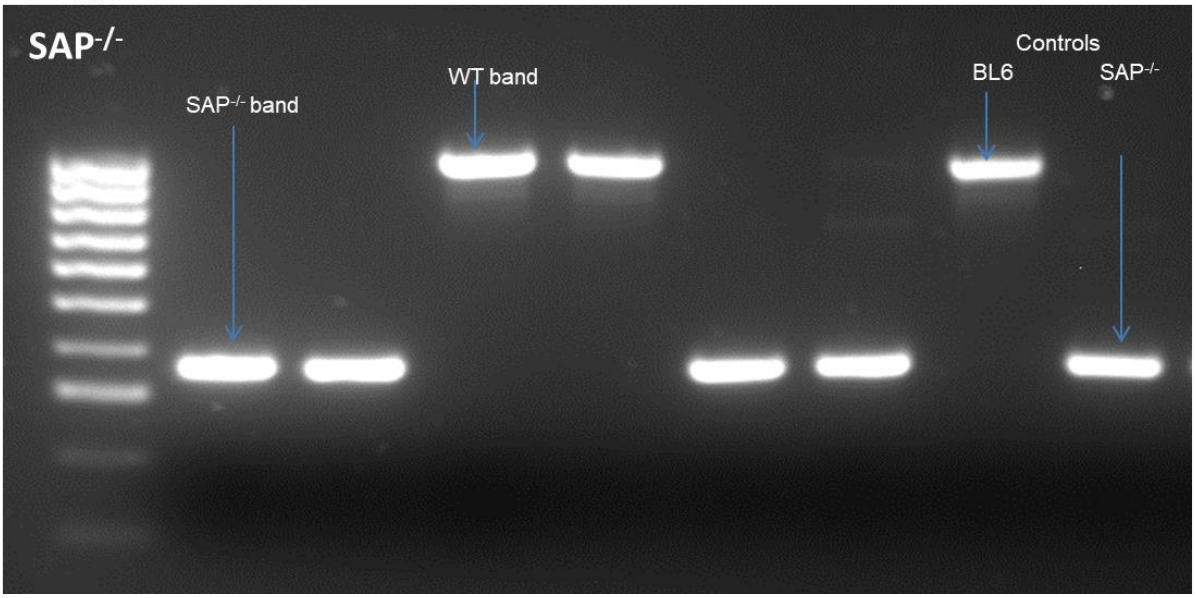


Figure 2.2. DNA gel electrophoresis images of various animals using the conditions and primers as described in table 2.9, confirming the presence of respective bands for the animal of interest.

a. Presence of a DNA band at 268bp with absent WT (BL6) band confirms the presence of homozygous status of bm12.
b. Presence of a DNA band at 520bp with a lack of WT (BL6) band confirms the homozygous status of $DM^{-/-}$ animals
c. Presence of a DNA band at 350bp with a lack of WT (BL6) band at 1000bp confirms the homozygous status of $SAP^{-/-}$ animals.

Recovery of fractionated DNA

Gels were visualised using a Trans illuminator (Syngene UK, Cambridge, UK) and the DNA band of interest was excised using a sharp clean scalpel and cleaned using QIA quick Gel Purification Kit (Qiagen Inc.).

2.2.10.2. Analysis of DNA by DNA sequencing

DNA sequencing of PCR products and plasmid DNA was carried out by the Source Bioscience Sanger sequencing facility, DNA sequences were analysed using the online ClustalW2 sequence alignment software provided by the European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI).

2.2.11. Recombinant DNA technology

2.2.11.1. Background and basic principle

It is a technique used in genetic engineering to combine DNA from different genomes. It involves the identification, isolation and insertion of gene of interest into a vector such as a plasmid or bacteriophage to form a recombinant DNA molecule that is capable of autonomous replication in a suitable host. The vector backbone can contain additional genes and sequences that may for example, facilitate expression of the insert DNA or help track uptake of the construct by a host cell.

I used this technique to deliver cDNA of Bcl-6 which is a master regulator of T_{FH} cells to a lenti-vector. So, I have had to construct the cDNA of Bcl-6 in the lenti-vector and then co-transfect it with its packaging plasmids to facilitate expression of cDNA gene.

I carried out this part of my work under kind supervision of J Garland Zhao who developed this vector in house (Zhao and Lever, 2007)

2.2.11.2. General protocol

Isolation and identification of insert cDNA

Bcl-6 pMIG-GFP vector plasmid (parent vector) was kindly gifted by S Crotty (Johnston et al., 2009). I isolated Bcl-6 cDNA by PCR amplification of the insert by using primers that had Bcl-6.BAMHI restriction sites at the end (sequences shown in table 2.8). Briefly, acquisition of cDNA of Bcl-6 involved the following step.

- Firstly, transformation of competent E. coli with PCR product was carried out (see below)
- Secondly, only peripheral and discrete colonies were selected and picked for further culture (see below).
- Thirdly, extraction and purification of DNA from the selected colonies was done by using Qiagen Mini Prep Kit (see below) and the presence of Bcl-6 band on gel electrophoresis was confirmed. In addition, Bcl-6 sequences was further confirmed on DNA sequencing (section 2.2.11.2)

Introduction of insert DNA into vector

After acquision of cDNA of Bcl-6, it was ligated with lentiviral expresser plasmids. All restriction enzymes were purchased from New England Bio Labs Inc. Both insert and vector DNA were treated with BAMHI restriction enzyme to get sticky ends. Thereafter, to prevent permanent phosphodiester linkage of any self-annealed vector molecules, 5' terminal phosphate groups were removed using the enzyme calf intestinal phosphatase (CIP). The insert was not dephosphorylated and therefore only vector molecule carrying the insert could be permanently re-circularised by DNA Ligase.

Digestion of vector using BAMHI:

30µl of viral vector

10µl of BAMHI-HF

10µl 10xTAS buffer

20 µl 5xBSA

30 µl H_2O

This mixture was incubated for 3hrs at 37°C. Then, I precipitated the DNA by incubating digestion mixture with 10µl of 3M sodium acetate (pH5.2) and 200µl 0f 100%ethanol at -80°C for 30 minutes. The mixture was then centrifuged at 15000rpm for 15minutes at 4°C followed by a wash with 70% ethanol and centrifuged again at 15000rpm for 15min at room temperature. Pellet was then air dried and dissolved in RNAase free water. This vector was dephosphorylated using CIP as manufacturer's instructions and then digested and dephosphorylated vector was purified using QIAquick PCR Purification Kit (Qiagen Inc.)

However, insert was PCR amplified using BAMHIBcl-6 forward and reverse primers and amplified PCR product was then digested with BAMHI-HF according to the manufacturer's instructions. Digested transgene was then isolated using Agarose gel electrophoresis and the QIAquick Gel Purification kit (Qiagen Inc.).

During DNA ligation, the insert was incubated with the plasmid backbone in 1:1, 2:1, 3:1 and 5:1 molar ratios with 1U T4 DNA Ligase (New England BioLab Inc.). The mixture was incubated at room temperature for 1hr.

Transformation of competent E. coli with vector

Recombinant vectors were amplified in the MAX Efficiency DH10B One Shot competent E. coli cells (Invitrogen, Life technologies Inc.). A 50µl aliquot of competent cells was thawed and incubated on ice with 1-5µl of the ligation reaction, or 5-10ng of purified plasmid for 30minutes. Cells were subjected to precisely 30seconds of heat treatment in a 42°C water bath, and transferred back immediately to ice. Cells were then recovered with 250µl of sterile SOC medium (Invitrogen, Life technologies Inc.) at room temperature and incubated at 30°C for 90minutes with vigorous shaking. The culture was evenly plated on pre-warmed LB Agar plated supplemented with 50µg/ml Ampicillin (LB/Amp plate) to facilitate selection. The plates were incubated at 30°C for 16-48hrs. The lower incubation temperature of 30°C was used to minimise the incidence of recombination events during bacterial replication of the relatively large mammalian expression vectors.

Selective expansion of recombinant vector

Single bacterial colonies were cultured for 16-24hrs at 30°C with vigorous shaking, in 3ml sterile LB broth supplemented with 50µg/ml of Ampicillin (LB/Amp). For a large scale DNA preparation, colonies carrying the plasmid of interest were further sub-cultured by inoculation of 1ml of bacterial culture in 500ml LB/ Amp medium for 24-48 hrs at 30°C with vigorous shaking.

Purification of recombinant vector

Bacterial cultures were centrifuged to obtain transformed cells; these were subjected to alkaline lysis and plasmid DNA was purified on a silicon resin according to the manufacturer's instructions with QIAprep MiniPrep or Qiagen Plasmid Maxi prep kits (Qiagen Inc.) DNA was eluted in nuclease free water (Ambio, Life technologies Inc.). The purity and approximate plasmid concentration of the preparation was checked using the Nanodrop spectrophotometer (Thermo Fisher Scientific Inc.) The presence of insert in LV expressor plasmid was confirmed by gel electrophoresis (figure 2.3) as well as by DNA sequencing.

Genetic screening of purified recombinant vectors

Restriction endonuclease cleavage of the purified plasmids was carried out by incubation of 1µg of vector DNA for 30minutes at 37°C and the size of resulting fragments was

determined using agarose gel electrophoresis. The restriction enzymes used and fragment sizes are given in table 2.10. All restriction enzymes were purchased from New England BioLab Inc.

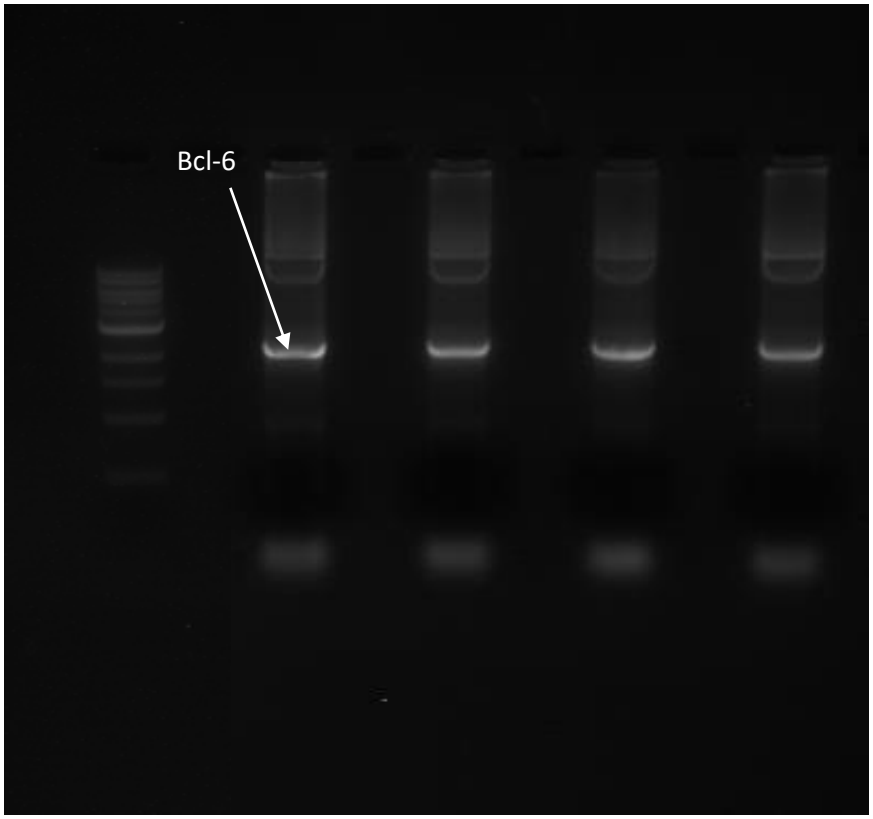


Figure 2.3. Gel electrophoresis of Bcl-6 LV vector confirming the Bcl-6 band at 2.1 kbp using BamHIBcl-6 forward and reverse primers.

Table 2.10. Restriction enzymes fragmentation of DNA backbone

DNA	Restriction enzyme	Fragment size
pCMV-EmGFP-BCL6	BAMHI	2.1kbp; 10kbp
pCMVΔR8.9	Xho1; EcoR1	10.4kbp; 1.6kbp
pCMV-VSV-G	Xho1	5.3kbp; 1.6kbp
pRSV-Rev1	Xho1; Bg1II	3.6kbp; .6kbp

Table 2.9. Restriction enzymes fragmentation of DNA backbone

2.2.12. Plasmid constructs

2.2.12.1. Background

Lentiviral vectors are a subtype of retroviruses and are highly efficient in infecting dividing and non-dividing cells; they have low immunogenicity and achieve long term stable expression of transgene in the host.

Virions are composed of three structural genes-*gag, pol*, and *env*-that code for the protein necessary for viral integration and replication; two regulatory genes-*tat* and *rev* and accessory genes- *vif, vpr, nef* and *vpu* which are associated with virus infectivity and pathogenesis. The viral genome is flanked by long terminal repeats (LTRs) that is essential for integration of the viral genome into the host and thereafter also act as a part of the promoter for viral gene transcription. A cis-acting Psi-sequence found near the 5' end of the genome is also necessary for packaging replicated viral RNA into virus capsids to sustain infection in the host.

My work used a human immunodeficiency virus-type 1 (HIV-1) derived third generation vector as has been used by Zhao et al (Zhao and Lever, 2007; Zhao et al., 2005; Zhao et al., 2002). This is regarded as a safe vector as most of the accessory genes are deleted and there is no tat in the regulatory gene. Third generation lentiviral systems are considered safer than second generation systems, however, are more difficult to use because they require transfection with four separate plasmids in order to create functional lentivirus vector.

The plasmids packaging systems in this vector are from two plasmids; one plasmid contains gag and pol and second provides rev. To broaden tropism of the vector, heterologous envelope from the vesicular stomatitis virus is used and its transgene is provided in a vesicular stomatitis virus G glycoprotein (VSV-G) expresser plasmid. These plasmids are replication deficient due to complete removal of Psi packaging element and LTRs from the plasmids. A transfer plasmid carries the transgene and reporter gene of interest together with the cis-acting genetic sequences that allow packaging and integration of the transgene, and provides the genetic material for the viral vector; notably promoter enhancer region in the 3' LTR of the transfer plasmid is partially deleted such that the reverse-transcribed pro-viral 5'LTR is transcriptionally inactive, preventing subsequent replication or mobilisation

following transduction. Following co-transfection of these vectors into an appropriate cell line, pseudoviral particles carrying the gene of interest are produced.

2.2.12.2. Lentiviral plasmids in the third generation packaging system

Plasmids vectors carrying the lentiviral genes were a kind gift from Prof. Andrew Lever and Dr. Jing Garland Zhao in the department of Medicine, University of Cambridge. All plasmids carried genes for Ampicillin resistance. These plasmids are discussed below.

Transgene expresser plasmid

The transgene expresser plasmid carries the transgene that will be integrated into the host genome. The transgene and reporter GFP gene are co-expressed from a bi-cistronic expression cassette with an internal spleen focus-forming virus promoter and the woodchuck hepatitis virus post transcriptional regulatory element. The vector was modified to incorporate transgenes for Bcl-6 within the expression cassette.

The plasmid contains the cis-acting Psi packaging element and the 5'LTR of the original provirus for efficient integration of the transgene, but has a 130 base pair deletion in U3 region of 3'LTR. It lacks most structural, regulatory and accessory genes from the HIV-1 sequence except 350 base pairs of *gag* sequence and rev response element (RRE) from the *env* sequence to increase packaging efficiency. A central polypurine tract (cPPT) that has been shown to improve transcription and transduction efficiency, has also been introduced upstream of the transgene. Bcl-6 transgene expressor plasmid is shown below in figure 2.4.

Vector name	SIN-SFFV-Bcl6-IRES-EGFP
Vector size	10293 bp
Insert size	2124 bp
Antibiotic	Ampicillin

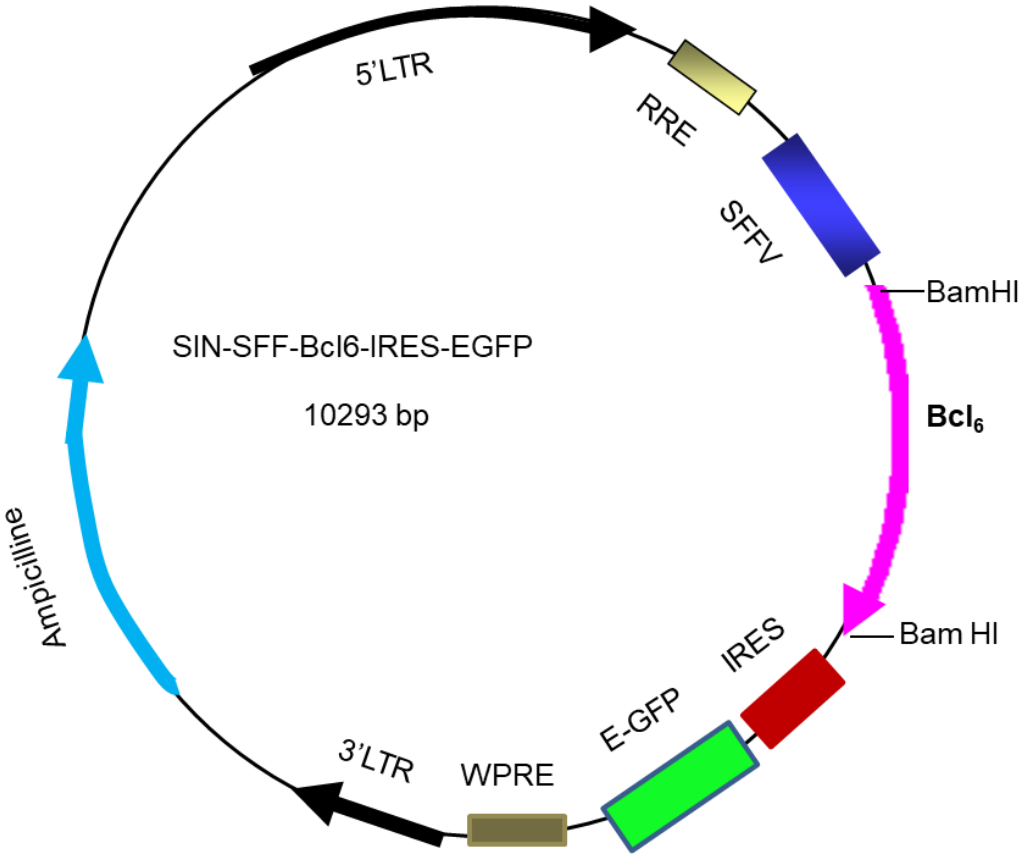


Figure 2.4. Schematic figure for Bcl-6 transgene expressor plasmid, whereSIN=Self-Inactivating 3' Long Terminal Repeat (LTR

SFFV=Strong internal promoter of SFFV (Spleen focus forming virus)

PRE= Post-transcriptional regulatory element

IRES= EMCV internal ribosome entry site

RRE= Rev responsive element

Bcl-6= B cell lymphoma 6 (master regulator of T_{FH} cells)

Bam HI= Restriction enzyme

E-GFP= Enhanced green fluorescent protein

WPRE= Woodchuck Hepatitis Virus Post transcriptional Response element

Gag-pol packaging plasmids (pCMV ΔR8.9)

The plasmid carries the HIV gag and pol structural genes, expressed by human cytomegalovirus immediate-early promoter (pCMV) and a Simian virus 40 (SV40) polyA tract. Majority of the *env* sequences has been deleted except for RRE to increase packaging efficiency.

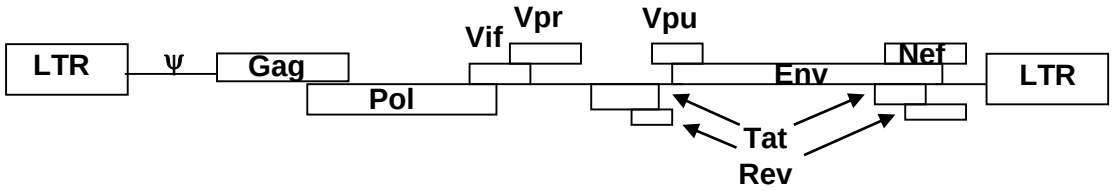
Rev expresser plasmids (pRSV-Rev)

This plasmid codes for the rev regulatory protein under control of the Rous Sarcoma virus (RSV) promoter and a SV40polyA tract. This protein can bind to RRE in the transgene expresser plasmid and pCMV ΔR8.9 plasmids, thus regulates virus RNA transport and splicing.

VSV-G expresser (pCMV-VSV-G)

This plasmid codes for the VSV-G proteins which is expressed by pCMV promoter and SV40polyA tract. The VSV-G envelope broadens the tropism and provides stability to the vector, thus allows particle concentration by ultracentrifugation.

i.



ii.

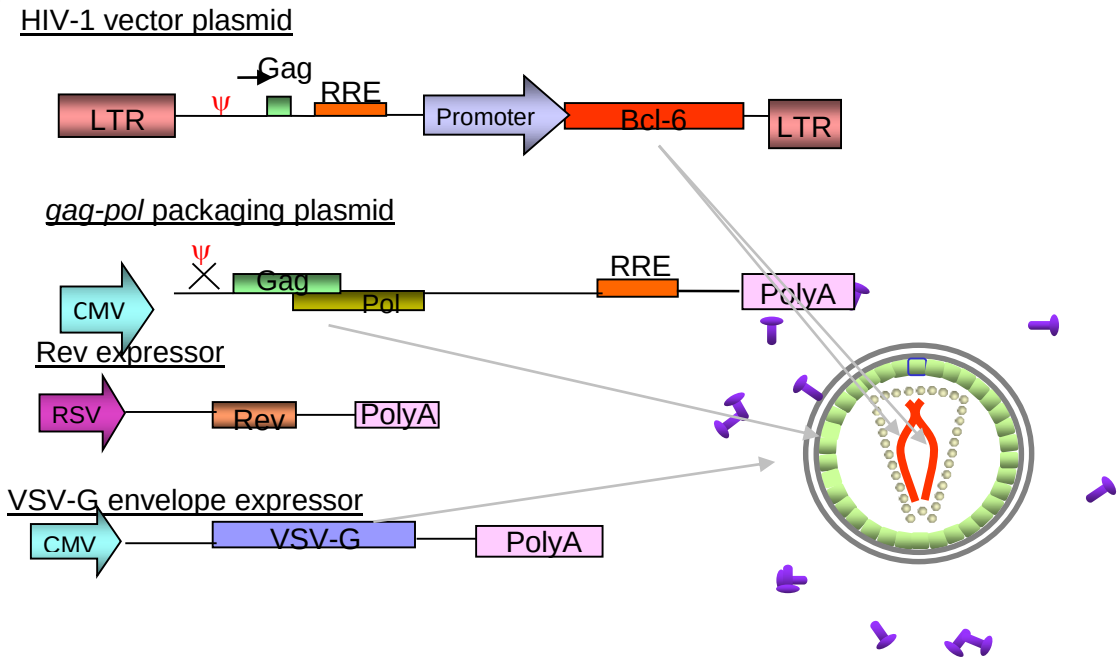


Figure 2.5. This figure has been adapted from J Garland Zhao's book (Zhao and Lever, 2007).

i. The genomic organisation of the lentivirus.

ii. The "third generation" of HIV-1 vector constructs with Bcl-6 as a transgene of interest.

2.2.13. Vector production and transduction

2.2.13.1. Background and basic principle

Pseudotyped vectors were generated by transfection of plasmid DNA into 293T cells using a modified calcium phosphate method (Chen and Okayama, 1988) and shown below (figure 2.4). Although the mechanism is not yet clear, it is believed that transfected DNA enters the cells by endocytosis and subsequently transfers to the nucleus. Since Graham and Van de Eb first established this methods in 1973 (Graham and van der Eb, 1973), many minor modifications of the procedure have been described. Among them Chen and Okayama's modified transfection protocol is considered to be highly efficient in achieving stable transformation of mammalian cells with supercoiled plasmid DNA (Chen and Okayama, 1988). In their modified calcium phosphate transfection protocol, DNA-calcium phosphate co-precipitate is formed gradually onto the cells in the tissue culture medium during prolong incubation (16-18 hours) under controlled conditions of pH 6.95 and 3% CO_2 tension.

2.2.13.2. Protocol

2.2.13.2.1. Mammalian cell cultures

HEK293 cells were cultured in full culture medium (DMEM supplemented with 2mM L-glutamine, 100IU/ml, penicillin, streptomycin, 10%FCS and Sodium Pyruvate). Cells were passaged by incubation with Trypsin-EDTA for 1 min at 37^0C water bath or till cells visibly detached under low power magnification.

2.2.13.2.2. Production of viruses

Setting up the plates at day 1

I split the exponentially growing 293 T cells and seeded them into 10-cm culture dishes at the 40-50 % confluence with 9 ml of DMEM supplemented with 10 % FCS and 1ml of split cell mix drop wise onto the plates and swirl gently. Make sure the cells are evenly dispersed in the culture dishes and then the cells are cultured overnight in the humidified 37^oC incubator in an atmosphere of 5 % CO_2. Plates will be harvested in multiples of 3, so maximum of 18 plates, one being a transfection control plate.

Transfection at day 2

Early afternoon, (2-3 hours before transfection) I aspirated off old media and added 9ml fresh medium and returned the plates to incubator. Late afternoon, I made transfection cocktail in 7ml bijous, 1 bijou per plate as follows and shown in figure 2.6.

737µl H_2O

737µl 2xBBS

10µl plasmid of interest

6.5µl pCMV ΔR8.9.

3.5µl VSVG

2µl Rev

72µl $CaCl_2$

I added H_20 and 2xBBS to each bijou, then DNA and finally $CaCl_2$. Each bijou was flicked gently as $CaCl_2$ is added, then left undisturbed for 20minutes. Transfection control is performed in the same way but with 7µl GFP as the only DNA. After 20 minutes, bijous were flicked again and added to the plates drop wise and swirled gently and returned to incubator for 12-16 hours.

Changing the medium and moving the plates to category 2 laboratory at day 3

Early morning, I aspirated off old medium and replaced with 12ml fresh DMEM medium (DMEM, Penicillin, Streptomycin, 10 %FCS, L/ Glutamine and sodium pyruvate) and transferred thee plates to category 2 laboratory in department of virology incubator.

Examination of the plates under UV at day 4

I examined the control plate for GFP expression under UV.

Harvesting the virus at day 5

It should be performed in category 2 laboratory hood.

Conditioned supernatant carrying the lentiviral particles was collected from three plates into a 50ml syringe and filtered through a 0.45µm filter into Beckman Ultra-clear centrifugation tubes to concentrate viral particles. It may be necessary to use more than one filter, but if more than three filters are required per 30ml of supernatant, then viral

production will probably be poor, and it may not be worth continuing. Each tube was topped up with few mm from the top with extra DMEM to prevent the tube buckling in the centrifuge. I placed the tubes in the swing buckets for SW32 rotor, and balanced to 0.05g (bucket 1 balances with bucket 4, 2 with 5 and 3 with 6- this is very important to double check these before starting centrifugation). Then buckets were attached with the rotor and spun @ 25000 for 2- 2.5 hours. The viral pellet was resuspended in 300µl 1% BSA in PBS; a 10µl aliquot was removed for testing and the remaining virus preparation was stored -80°C.

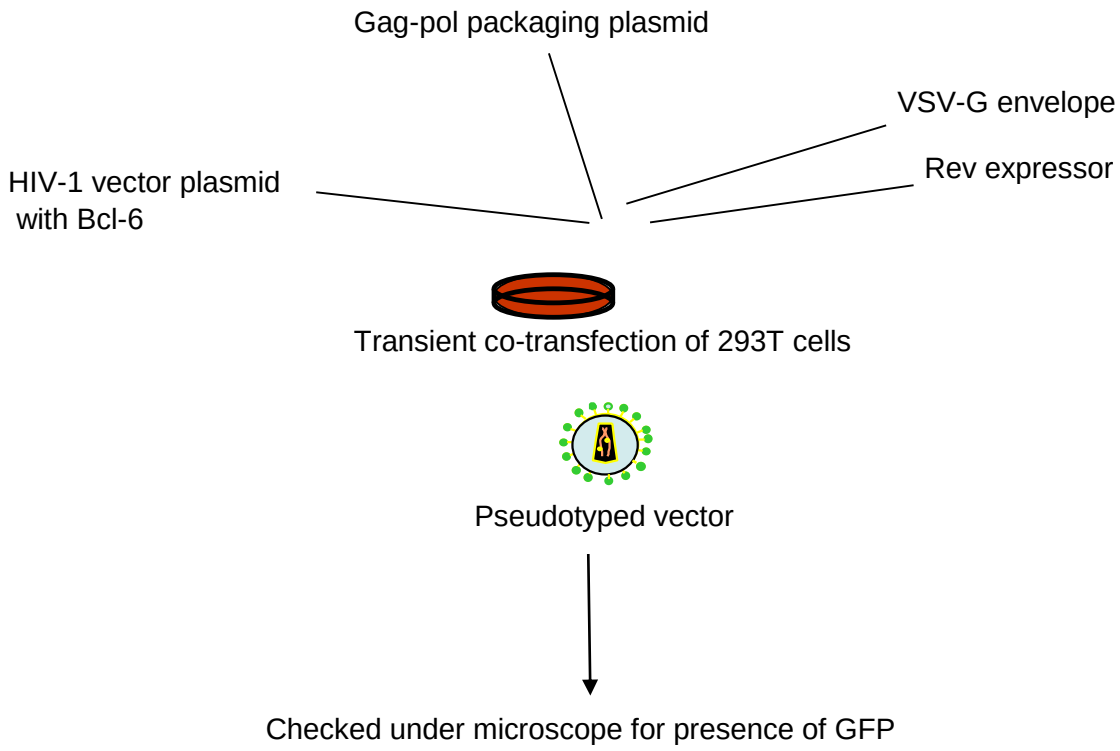


Figure 2.6. Generation of lentiviral vector by transient co-transfection of 293 T cells, adapted from Zhao et al (Zhao and Lever, 2007)

2.2.14. Transduction of murine CD4 T cells with Bcl-6 vector

This was adapted from Johnson et al (Johnston et al., 2009) and McCausland et al (McCausland et al., 2007).

Purification of CD4 T cells

Single cell suspension of splenocytes were resuspended in 750µl of MACS buffer with 100µl of CD4 magnetic beads and left on ice for 20 minutes. After washing and resuspension of cells in 500µl of MACS buffer; the cells were passed through the auto MACS machine using possels program and positive fraction was collected. Purified CD4 T cells were washed, resuspended in full medium (RPMI supplemented with 10% FCS, 100U/ml Penicillin/streptomycin, 2mM L-Glutamine (Gibco) and mouse IL-2 (50U/ml) and used for activation of CD4 T cells.

Activation of CD4 T cells

$8x10^4$ purified CD4 T cells in a volume of 100µl per well were placed on 96 well tissue culture plate. Then CD23/CD28 mouse T activator dynabeads (life technologies) were washed with FACS buffer and resuspended in full medium and added to CD4 T cells plate in a way to obtain a bead to cell ratio of 1:1. The plates were incubated in a humidified CO2 incubator at 37°C for 24 hours.

Transduction

Activated CD4 T cells were transferred to 12 well culture plate which was precoated with polybrene (10ng/ml) and Bcl-6 vector with multiplicity of infection (MOI) from 1-3 and control lentiviral vector without Bcl-6 GFP. These cells were then spun at 1200rpm for 90 minutes at 4°C with murine IL-2. Cells were washed and medium was replaced with minimum medium (RPMI supplemented with 1% FCS, 100U/ml Penicillin/streptomycin, 2mM L-Glutamine and 50U/ml of mouse IL-2) and left in humidified CO2 incubator at 37°C for 24 hours. After 24 hours, minimum medium was replaced with full medium and left in incubator for an additional 72 hours. Cells were then examined under microscope for fluorescence indicating the transduction of cells. It is important to note here that not all the bm12 CD4 T cells would have been transduced with Bcl-6 vector.

Delivery to animals

After confirmation of GFP under microscope in experimental wells and absence in control LV vector wells on bm12 CD4 T cells, cells were washed and beads were removed and cells ($2x10^6$ CD4 T cells) were injected intravenously into mice. As I mentioned above, in the experimental group, mixture of both transduced and non-transduced bm12 CD4 T cells would have been transferred.

2.2.15. Statistical analysis

Data were presented as mean ± S.D. where appropriate. Mann Whitney tests were used for analysis of non-parametric data. Two-way ANOVA was employed for comparison of antinuclear and anti-Kd antibody responses. Graft survival was depicted using Kaplan-Meier analysis and groups compared by log-rank (Mantel-Cox) testing. Analysis was conducted using Graph Pad 4 (Graph- Pad Software, San Diego, CA, USA). Values of $P < 0.05$ were considered significant. Furthermore, p values were substratified as $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ where applicable.

Chapter 3

Characterisation of germinal centres in a model of chronic rejection (bm12 to BL6)

3.1. Introduction

The experimental work in this thesis is an extension of our previous work, examining chronic heart allograft rejection in the murine bm12 to C57BL/6 (BL6) strain combination (Win et al., 2009). The bm12 strain is a naturally occurring mutant of BL6 that differs by only three amino acids in the third hypervariable region of the peptide binding groove of the MHC class II, I-A^b. This creates the so-called I-A^{bm12} class II molecule in the bm12 strain. The amino acid changes are at codon 67 (isoleucine in BL6 replaced by phenyalanine in bm12), codon 70 (arginine in BL6 replaced by glutamine in bm12) and codon 71 (threonine in BL6 replaced by lysine in bm12) (Hansen and Tse, 1987; McKenzie et al., 1979; Mengle-Gaw et al., 1984; Tse et al., 1985). The BL6 and the bm12 strains only express one MHC class II antigen, because the I-E antigen is not expressed due to deletion of the Eα gene. Hence, the two strains are mismatched at MHC class II but are otherwise identical at the MHC class I and minor loci.

3.1.1. Mechanisms of rejection in this model

Due to minimal mismatch between these two strains, the responder frequency of donor T cells that interact with the I-A^b alloantigen was much less than that found in completely MHC mismatched groups. Suchin et al (Suchin et al., 2001) found a responder frequency of 2.5% compared to the 21% in the complete MHC mismatch group. Similar results were reported by Busser et al (Busser et al., 2003). Nevertheless there are enough anti-bm12 T cells within the BL6 repertoire to elicit acute rejection of bm12 skin allografts by BL6 mice (Nagano et al., 1997; Stuart et al., 1984). Many groups have reported chronic rejection of bm12 donor heart allografts in BL6 recipients (Gao et al., 2000; Sayegh et al., 2003; Win et al., 2009).

Rejection in this model appears to be dependent upon recipient T cells. The evidence comes from Furukwawa et al study (Furukawa et al., 2004) in which development of AV in the bm12 donor heart grafts was observed upon transplantation into RAG-$1^{-/-}$ recipients following reconstitution with recipient T cells, even in the absence of B cells (Furukawa et al., 2004). This AV was dependent upon IFN- γ, as no AV was observed when IFN- $\gamma^{-/-}$ T cells were transferred into RAG-$1^{-/-}$ recipients (Furukawa et al., 2004). Interestingly, AV markedly attenuated when bm12 allografts were transplanted into IFN- $\gamma^{-/-}$ recipients (Nagano et al.,

1997). Similar results have been reported by other groups as well in which they have shown that $CD8^+$ T cells, activated via the CD40-CD40L pathway, mediated the progression of AV via their cytotoxic activity and secretion of IFN-γ (Fischbein et al., 2000; Fischbein et al., 2001). In keeping with these studies, AV significantly reduced in CD8 T cell knock out recipients (Schnickel et al., 2004) suggesting that CD8 T cells playing a role in allograft rejection in this model. Recently, Ishii et al investigated the role of CD8 and CD4 T cells in this model by transplanting bm12 kidney allografts into $CCR5^{-/-}$ BL6 recipients (Ishii et al., 2014). Although, numbers of CD4 T cells infiltrating bm12 allografts were nearly identical in BL6 and $CCR5^{-/-}$ BL6 recipients, there was an intense infiltration of CD8 T cells in allografts transplanted into $CCR5^{-/-}$ BL6 recipients, that was detectable 1 week after transplantation and gradually increased thereafter. The CD8 T cells induced to bm12 allografts in CCR5-deficient recipients were reactive to donor $I\text{-}A^{bm12}$ peptide presented by class I MHC molecules (indirect CD8 T cell response). Their results demonstrated unique CD8 T cells specificities against disparate $I\text{-}A^{bm12}$ peptides which is capable of rejecting the donor kidney allografts in the absence of CCR5 expression through indirect pathway (Ishii et al., 2014). However, earlier studies (Valujskikh et al., 2006) have contradicted Ishii et al findings. Valujskikh et al experiments suggest that CD8 T cells reactive to self-restricted allopeptide are non-pathogenic bystanders within the alloimmune response to fully MHC-disparate heart grafts. By using a defined host-restricted, minor transplantation Ag (HY, male antigen), they showed that preactivated Ag-specific CD8 T cells did not influence the kinetics of acute cardiac allograft rejection, did not influence the development of chronic graft injury, and did not adversely affect costimulatory blockade-induced prolongation of graft survival (Valujskikh et al., 2006).

Further examination of rejection kinetics in this model has shown that both direct and indirect pathways of CD4 T cell allorecognition are playing a role in chronic rejection. Thus, BL6 CD4 T cells can recognise intact $I\text{-}A^{bm12}$ molecule directly on donor cells or, alternatively, as processed allopeptide presented by self MHC class II ($I\text{-}A^b$) on BL6 APCs (indirectly). The evidence for the role of direct pathway is derived from the observation of development of AV in bm12 donor cardiac allografts upon transplantation into H-2M BL6 (Ardehali et al., 2002). H-2M BL6 mice express normal levels of MHC class II but possess a defect in peptide loading. Hence, recipient CD4 T cells can only recognise $I\text{-}A^{bm12}$ by the direct pathway. However, the contribution of the indirect pathway is demonstrated by the prolonged

survival and diminished vasculopathy of the bm12 cardiac allografts upon transplantation into TCR-transgenic ABM recipients. ABM mice have a monoclonal population of CD4 T cells that recognise the bm12 antigen by the direct pathway (Sayegh et al., 2003). Surprisingly, ABM recipients reject bm12 skin grafts, perhaps reflecting that skin grafts are generally more immunogenic that other vascularized organs. Further evidence about CD4 T cell indirect alloreactivity in this model is suggested by the more rapid rejection of bm12 allografts that occurs when transplanted into BL6 recipients previously primed with synthetic I-A^{bm12} peptide that incorporates the disparate amino acids (Win et al., 2009). It is not clear how indirect pathway CD4 T cell will mediate allograft damage because of their inability to bind with the graft cells in an antigen specific manner due to lack of their epitope on the allograft. It is more likely that these indirect pathway CD4 T cells will be acting as a helper to either cytotoxic CD8 T cells or B cells to generate antibodies.

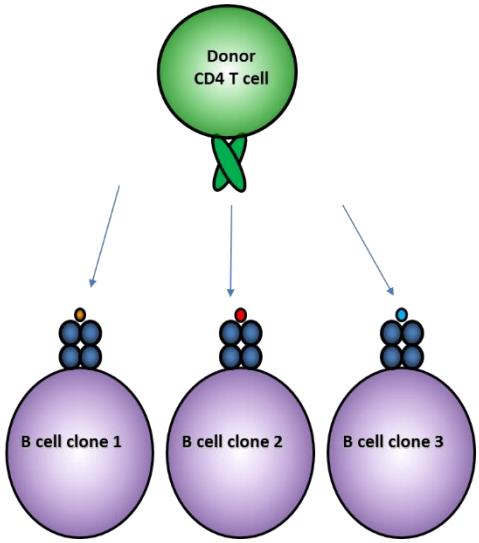
3.1.2. Development of autoantibody in this model

Previous work in our department (Win et al., 2009) and by others (McKenzie et al., 1979) has shown that alloantibody responses against the I-A^{bm12} donor antigen are not generated in BL6 recipients of bm12 heart allografts. This most likely reflects the absence of conformational epitope on the I-A^{bm12} antigen for recognition by BL6 B cells, rather than an absence of the provision of essential CD4 T cell help, because immunisation of BL6 mice with F1 (bm12xH-2^k) splenocytes results in development of anti-H-2^kIgG alloantibody (indicating delivery of appropriate T cell help), and yet, anti-I-A^{bm12} alloantibody is not generated (Win et al., 2009). Instead BL6 recipients of bm12 heart allografts developed long lasting anti-nuclear IgG autoantibodies with germinal centre secondary follicles evident within the recipient spleen on hematoxylin and eosin–stained paraffin sections, indicating T cell dependent, humoral immunity (Win et al., 2009). The development of autoantibody was determined by hep-2 indirect immunofluorescence, with patterns of staining of the Hep-2 cells differing markedly between differing recipients. This implies that this humoral autoimmune response was directed against multiple different autoantigens (Win et al., 2009). In this model, we reported development of anti-chromatin, anti-dsDNA and anti-ssDNA autoantibodies (Win et al., 2009), while Mahesh et al (Mahesh et al., 2007) reported development of anti-vimentin autoantibody responses.

It is not immediately clear why bm12 allografts trigger humoral autoimmunity in BL6 recipients. Earlier work by Eisenberg et al. reported that adoptive transfer of bm12 splenocytes provoked autoantibody development and SLE like immunopathology in BL6 recipients (Morris et al., 1990a). He further suggested that the autoantibodies that are generated during chronic GVH phenomenon are the result of physical T-B cell interactions, rather than a consequence of secretion of nonspecific T cell-derived factors. Their experiments involved adoptive transfer of bm12 splenocytes into radiation mixed chimeras of bm12 and BL6 bone marrow elements which resulted in ANAs secreted only from BL6 B cells, despite the potential for bm12 B cells to be activated by bystander mechanisms (Morris et al., 1990b). Furthermore, the donor bm12 T cells activated the entire recipient B cell population, as demonstrated by the increase in size of the recipient B cells, along with uniform expression of higher levels of MHC class II and B7-2 (Sekiguchi et al., 2002). However, the development of anti-dsDNA autoantibodies was driven by the self-antigens (Sekiguchi et al., 2003). Sekiguchi et al (Sekiguchi et al., 2003) used two different heavy chain Ig site directed transgenes; 3H9 knock in (KI) and 3H9/56R KI and adoptively transferred bm12 splenocytes to these transgenic mice. Their previous work has shown that 3H9 KI produces wider repertoire of autoantibodies (Li et al., 2001) and 3H9/56R heavy chain KI gives rise to more specific anti-dsDNA antibodies (Chen et al., 1995). On analysis of autoantibodies, they found similar levels of anti-ssDNA in the 3H9 and 3H9/56R recipients but an increased levels of anti-dsDNA antibodies in 56R KI recipients. In 56RKI mice, the difference in increased levels of anti-dsDNA antibodies was due to the presence of the anti-DNA Ig sd-tg focused allogeneic T cell help on anti-dsDNA B cells. This was confirmed by hybridomas showing consistently high percentage of hybridomas that were anti-dsDNA positive in 56RKI^{+ve} group. Interestingly, most of the anti-dsDNA clones in the 56RKI^{+ve} set were not using the 3H9/56R sd-tg, hence the presence of this high frequency could not be directly attributed to the presence of anti-DNA H chain. Rather, the presence of high frequency of anti-dsDNAs producing hybridomas was due to the self-antigens which were selectively activating these cells in vivo. Genetic analysis of hybridoma panels of the rearranged Ig genes correlated this response with the generation of anti-dsDNA B cells through secondary rearrangements that replaced the site-directed transgene (sd-tg) with endogenous VH genes.

There may be two potential mechanisms which may be leading to the development of autoantibodies only. Firstly, autoantigens stimulate the toll like receptors, antinuclear autoantigens mimic toll-like receptor (TLR) ligands and once internalised, provide additional TLR signalling for antibody production (Leadbetter et al., 2002) and secondly, concurrent ligation of BCR by autoantigens result in the development of autoantibodies (Harper et al., 2016). The later possibility was confirmed by adoptive transfer of bm12 CD4 T cells to $TCR^{bd-/-}$ BL6 mice that are simultaneously immunised with adjuvant alone or with ovalbumin in adjuvant. Both groups developed autoantibody, but only those immunised with ovalbumin developed IgG anti-ovalbumin antibody. Harper further demonstrated that this phenomenon also applied to alloantigenic determinants that were expressed on the donor CD4 T cells; that these donor cells could provide help for alloantibody responses against their self-antigens. This occurred even though these CD4 T cells were, as expected, tolerant to their surface MHC antigens within the donor strain. Thus Harper's results indicated that although donor CD 4 T cells activate all the B cells, differentiation into antibody secreting cells requires concurrent ligation of the BCR (see Figure 3.1). This conforms the two signal hypothesis of B cell activation (Bretscher and Cohn, 1970).

i.



ii.

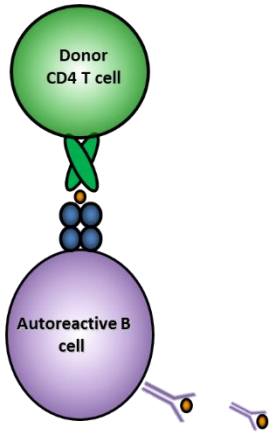


Figure 3.1. Two signal hypothesis of B cell activation in bm12 to BL6 model.

i. Donor CD4 T cells provide help to recipient B cells by GVH recognition of allogeneic MHC II on the B cells surface, irrespective of the peptide bound to MHC class II.

ii. Differentiation to an antibody secreting cell is limited to those B cells that simultaneously bind target antigen via their B cell receptor.
(Adapted from Harper Ines et al et al work) (Harper et al., 2016)

An effector role humoral autoimmunity in contributing to slowly evolving AV presumably requires long term production of autoantibody. In our previous studies (Motallebzadeh et al., 2012; Win et al., 2009), we have shown that autoimmunity triggered by bm12 allografts in BL6 recipients was long-lasting, with strong titres maintained up to three months post transplantation. It is generally accepted that long lasting humoral responses are the result of long-lived plasma cells (Manz et al., 1997; Slifka et al., 1998), which are in turn a product of germinal centres. (Good-Jacobson et al., 2010; Han et al., 1995; MacLennan, 1994; Takahashi et al., 1998; Vinuesa et al., 2009). Although Shlomchik et al have shown in an autoimmune prone mice model that EF antibody responses may result in deposition of plasmablasts in the spleen, and further, that these antibody forming plasma cells showed evidence of hypermutation, despite not being derived from a germinal centre (William et al., 2005a; William et al., 2005b). Nevertheless these antibody responses were not long-lived (William et al., 2005b).

The development and maintenance of these GCs is dependent on availability of help from T_{FH} cell (Crotty, 2011; Crotty, 2014; Crotty, 2015; Qi, 2012; Vinuesa and Cyster, 2011). The T_{FH} cell provides survival signals to follicular B cells for development of GC response that, ultimately, provide life time immunity. Conversely, there is emerging evidence that dysregulation of these T_{FH} cells may be a trigger in development of autoimmunity. This has been shown by Linterman et al in Roquin$^{san/san}$ mouse model in which aberrant help by T_{FH} CD4 T cells resulted in the development of lupus like pathology without any antigenic challenge. However, when SAP signalling was inhibited (which is thought to be important for the development of GCs) in Roquin$^{san/san}$ SAP^{-ve} mice, excessive T_{FH} cells or spontaneous GCs did not form, and lupus like pathology in the animals was ameliorated, thus confirming the role of T_{FH} cells for the development of autoimmune GCs. Others have also shown that aberrant T cell help can cause spontaneous GC formation and autoimmunity (Luzina et al., 2001; Vinuesa et al., 2009). Nevertheless, T cell help is critical for the development of long lasting germinal centres. Hence, I examined the development of germinal centres in bm12 to BL6 model, in particular to focus upon the T_{FH} subset of CD4 T cell.

3.2. Aims

Because the previous studies have not investigated the development of germinal centres in this model, the aim of this chapter is to characterise the germinal centre humoral responses and address the following questions:

1. Do germinal centres develop in BL6 recipients of a bm12 heart allograft?
2. If so, is the germinal centre response long lasting?
3. Are these GC responses mediated by T_{FH} cells?
4. Does the autoimmune response diversify in this model?

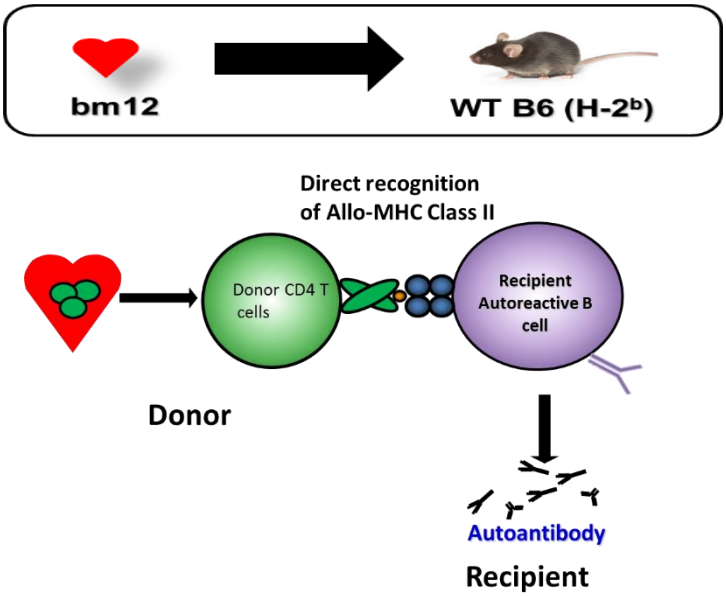
3.3. Results

3.3.1. Development of autoantibody

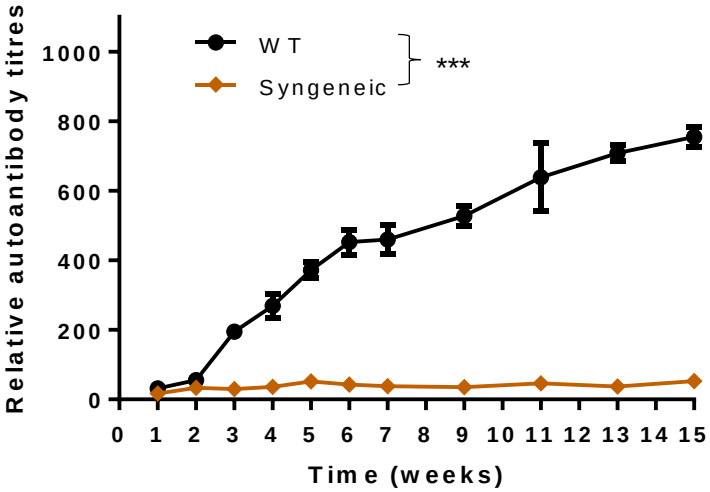
Before embarking to investigate development of germinal centres in this model, I decided to confirm the previous findings (Win et al., 2009) first by transplanting bm12 heart grafts into BL6 recipients and examining autoantibodies in the recipients' sera, assessing rejection kinetics of bm12 allografts and measuring development of allograft vasculopathy in donor allografts on explanation.

Analysis of recipients' sera by hep-2 indirect immunofluorescence staining demonstrated a long lived autoimmune humoral response (figure 3.1).

a.


bm12
WT B6 (H-2ᵇ)
Direct recognition
of Allo-MHC Class II
Donor CD4 T
cells
Recipient
Autoreactive B
cell
Donor
Autoantibody
Recipient

b.


Relative autoantibody titres
1000
800
600
400
200
0
W T
Syngeneic
***
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Time (weeks)

c.

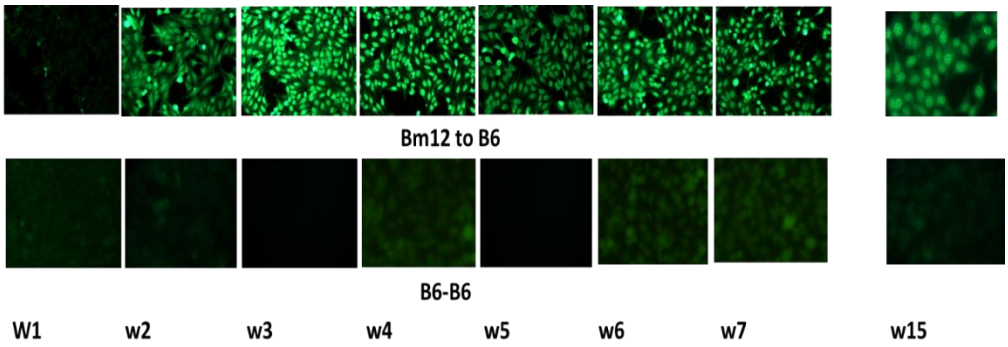

Bm12 to B6
B6-B6
W1
w2
w3
w4
w5
w6
w7
w15

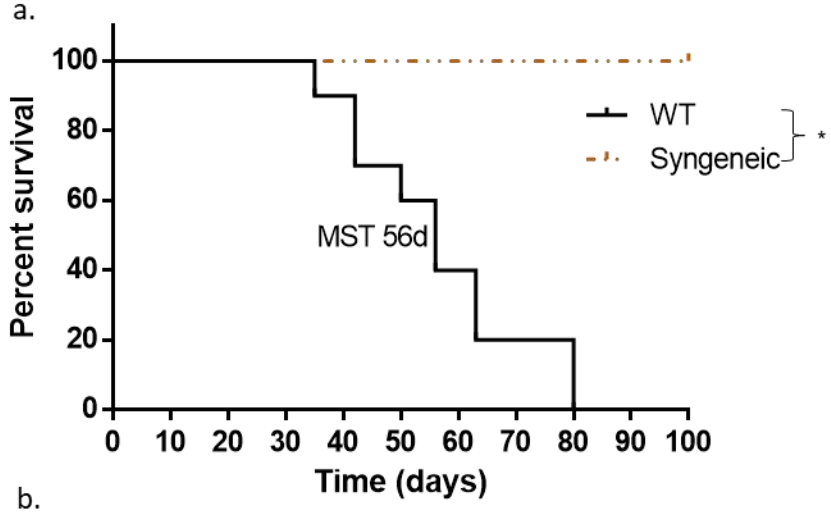
Figure 3.2. Development of antinuclear autoantibodies in BL6 recipients of bm12 donor heart grafts

a. Pictorial presentation of the model in which donor CD4 T cell directly interacts with recipient B cells through their MHC class II in a graft versus host fashion and provide help for the development of autoantibody.
b. Relative anti-nuclear autoantibody levels as measured by Hep-2 indirect immunofluorescence in BL6 recipients sera demonstrating development of long lasting autoantibody responses in BL6 recipients of bm12 allografts compared to BL6 recipients who received syngenic heart grafts ($p=<0.0001$, Two way ANNOVA). Data represent mean and SD of n = 4 mice per group.
c. Representative pictures of hep-2 indirect immunofluorescence slides for bm12 to BL6 versus syngenic grafts.

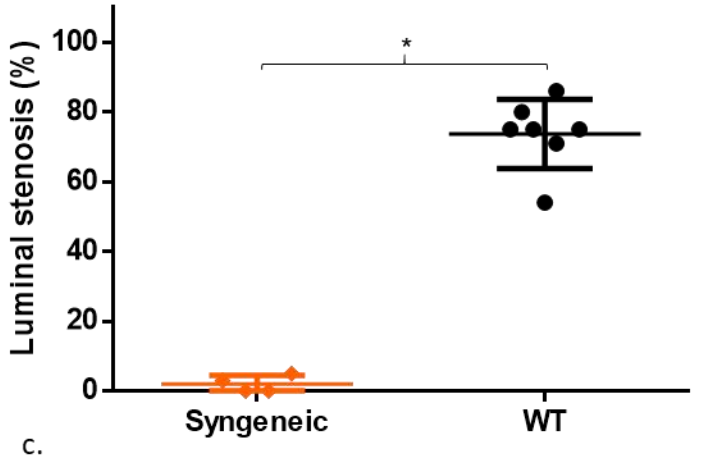
3.3.2. Graft survival and histology

Bm12 allografts were rejected in BL6 recipient slowly with median survival of 56 days compared to long term survival of syngeneic BL6 heart grafts (p=0.005, log rank)(n=10)(figure 3.3a). Rejection was associated with the development of AV in donor bm12 allografts, not observed in BL6 syngeneic grafts (mean luminal stenosis was 76±6 in bm12 to BL6 vs 2±2 in BL6 to BL6, p= 0.02) (figure 3.3b). This luminal stenosis was calculated as described in methods section (2.2.7.5) in the donor allografts explanted at week 7 post transplantation.

a.


Percent survival
100
80
60
40
20
0
0 10 20 30 40 50 60 70 80 90 100
Time (days)
MST 56d
WT
Syngeneic
*

b.


Luminal stenosis (%)
100
80
60
40
20
0
*
Syngeneic
WT

c.

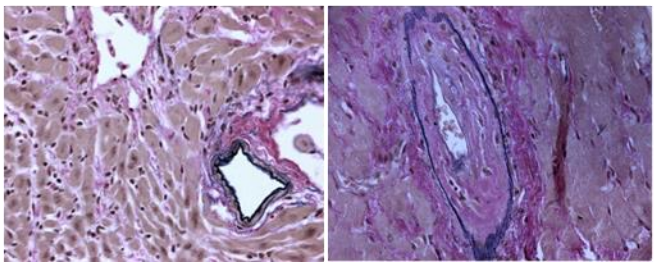


Figure 3.3. Development of allograft vasculopathy in bm12 donor grafts following transplantation into BL6 recipients

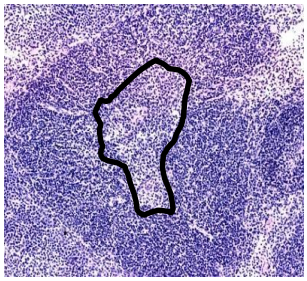
a. Kaplan-Meier survival curves of bm12 hearts transplanted into BL6 (n=5-10) and BL6 hearts transplanted into BL6 (log-rank test, p=0.003).
b. Development of significant allograft vasculopathy (% luminal stenosis) in donor hearts explanted from bm12 to BL6 group compared to syngeneic group at 7 weeks post transplantation (n= 4-7) (p= 0.02, Mann-Whitney test).
c. Representative figure for the development of AV, explanted hearts were stained with Elastic Van Gieson staining (EVG) in syngenic and bm12 to BL6 group (n= 4).

3.3.3. Histological assessment of recipient spleen sections for the development of germinal centres

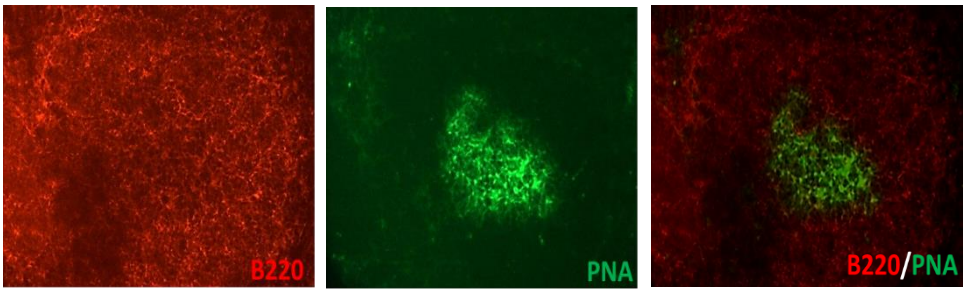
As bm12 allografts were rejected slowly in BL6 (WT) recipients with development of allograft vasculopathy and recipient sera demonstrated long lasting autoantibody response, the next step was to investigate how these autoantibodies are formed.

Win's original work in our department had reported that haematoxylin and eosin (H&E) staining of splenic sections from BL6 recipients seven weeks after transplantation with a bm12 heart allograft revealed classical light and dark zones, consistent with a germinal centre response (Win et al., 2009) but this was not explored further. Therefore, I performed immunohistochemistry (IHC) and immunofluorescence (IF) staining on recipients' spleen section to first confirm the development of GC response. As expected, on H and E staining of BL6 recipients splenic sections revealed dark and light zones characteristic of secondary follicles (Figure 3.4a). To characterise these further, I performed immunofluorescence staining on recipient splenic sections with GC-specific markers (PNA (Rose et al., 1980) and / or GL7 (Yusuf et al., 2010)).This enabled me firstly to formally quantify the number of GCs present within the spleen sections and secondly to identify presence of CD4 T cells within the secondary follicles, in the expectation that these cells would represent CD4 T follicular helper T (T_{FH}) cells (Garside et al., 1998; Yu and Vinuesa, 2010). Immunofluorescence staining of splenic tissue section from naïve animals was initially performed to optimise the staining protocol to be used throughout this thesis. An example of a positive GC and a negative GC is shown below in figure 3.4 b and c.

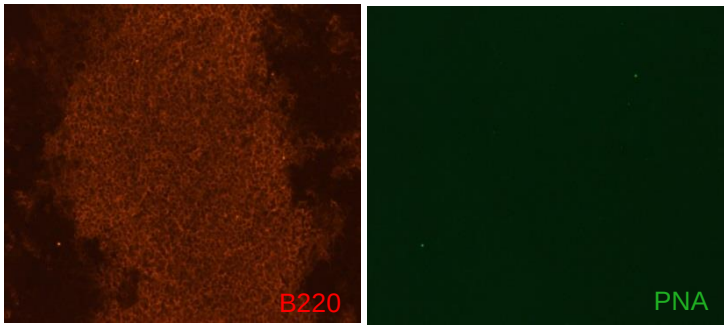
a.



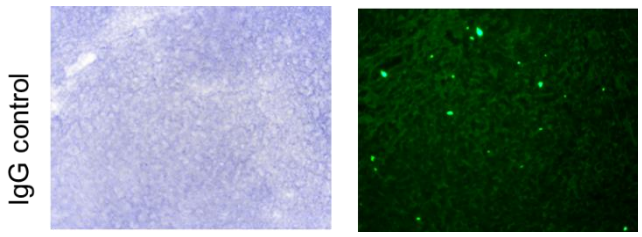
b. B cell follicle positive PNA staining co-localised



c. B cell follicle negative PNA staining



d.



e.

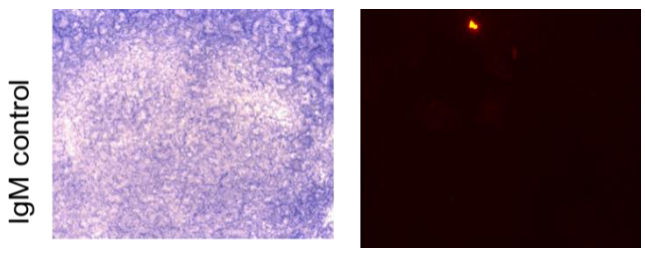
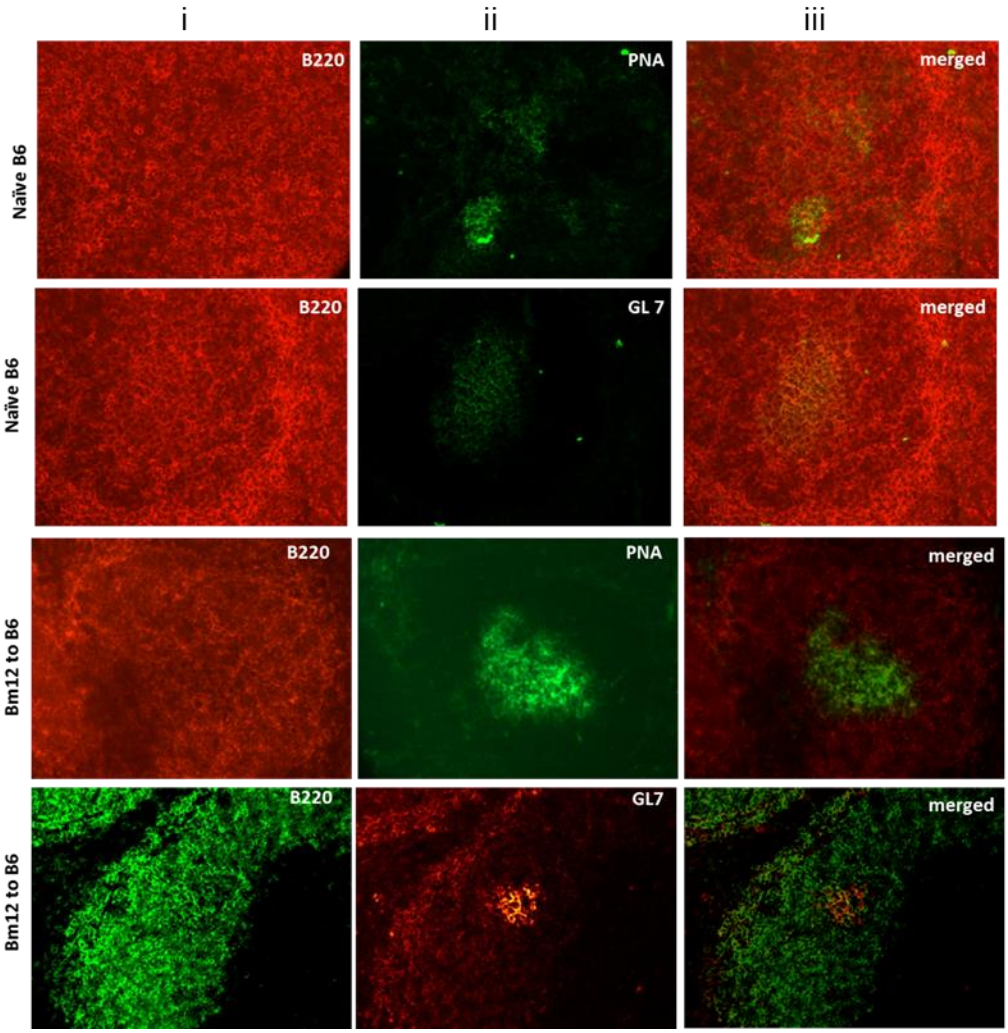


Figure 3.4. Immunofluorescence staining of spleen sections showing presence or absence of germinal centres. All the pictures were taken at 20x magnification

a. A representative image of dark zone and light zone in recipient B cell follicle on haematoxylin and eosin staining in splenic sections of BL6 recipients who received bm12 heart grafts. Staining was performed at 7 weeks post transplantation. Light zone characteristic of germinal centre is highlighted by black line.
b. A representative image of a positive germinal centre (GC) immunofluorescence staining as seen in BL6 recipient spleen sections of bm12 allografts. Staining was performed with anti-B220 antibody (Ab) and anti PNA Ab and co-localization of B220 and PNA highlighting the presence of GC.
c. A representative image of a negative GC where B cells did not develop into GC as seen in splenic sections of BL6 recipients who received syngeneic BL6 heart grafts Staining was performed at 7 weeks post transplantation with anti-B220 Ab and anti PNA Ab.
d. Isotype control for IgG, stained with Rat IgG2b kappa. This is an isotype control for B220 IgG.
e. Isotype control for IgM stained with Rat IgM. This is an isotype control for GL7 IgM antibody.

The germinal centres were quantified in spleen sections of BL6 recipients of bm12 and BL6 hearts by calculating the percentage of GC positive B cell follicles to total number of follicles (section 2.2.8.5). On quantification of GCs, a significantly high percentage of GCs was found in BL6 recipients of bm12 allografts compared to syngeneic controls at week 7 post transplantation ($p= 0.02$) (figure 3.5).

a.


i
ii
iii
Naïve B6
B220
PNA
merged
Naïve B6
B220
GL 7
merged
Bm12 to B6
B220
PNA
merged
Bm12 to B6
B220
GL7
merged

b.

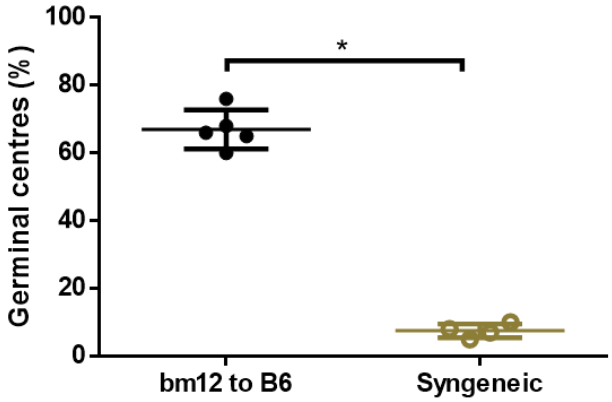

Germinal centres (%)
100
80
60
40
20
0
*
bm12 to B6
Syngeneic

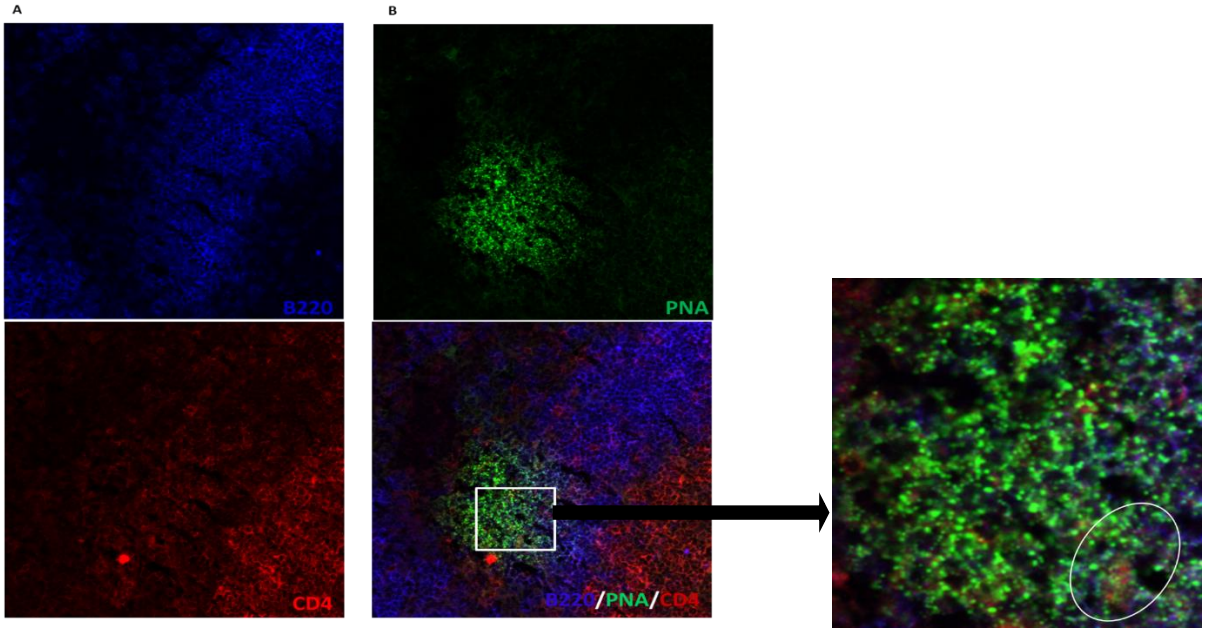
Figure 3.5. Quantification of germinal centres in BL6 recipients of bm12 allografts

a. Immunofluorescence staining of spleen sections showing presence of germinal centres in naïve BL6 and BL6 recipient of bm12 heart graft at week 7 post transplantation. Staining was performed with anti-B220 Ab (i panels) and anti PNA (ii panel) and anti-GL 7 Ab (ii panel). Co-localization of B220 and PNA (iii panel, merged) and B220 and GL 7 (iii panels, merged) highlighting the presence of GCs. All the pictures were taken at 20x magnification.

b. Quantification of germinal centres in BL6 recipients of bm12 allografts or syngenic BL6 heart grafts at week 7 post transplantation. This showed that more than 50% of the primary follicles differentiated into secondary follicles or germinal centres as demonstrated by PNA^+ and / GL 7^+ B220 follicles in the WT BL6 recipients of bm12 hearts compared to BL6 hearts (syngenic) at week 7. Data represent mean and SD of n = 4-5 mice per group, $*P < 0.05$, (Mann-Whitney test)**.**

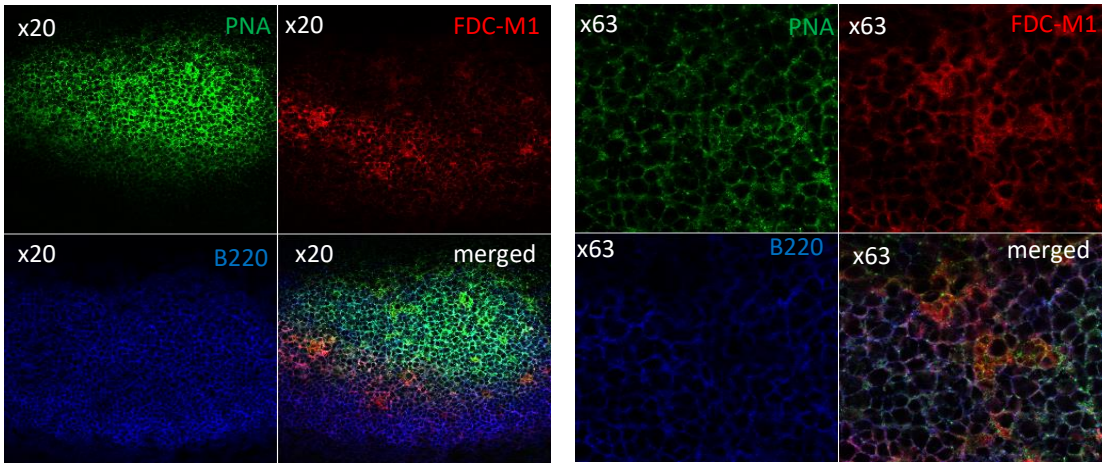
These GCs were further characterised by identifying follicular dendritic network within GC by staining with FDC marker FDC-M1 and localisation of CD4 T cells in GCs. CD4 T cells were found to be seen within secondary follicles as well as within germinal centres B cells (figure 3.6).

a.


A
B
B220
PNA
CD4
B220/PNA/CD4

b.

c.


x20
PNA
x20
FDC-M1
x20
B220
x20
merged
x63
PNA
x63
FDC-M1
x63
B220
x63
merged

d.

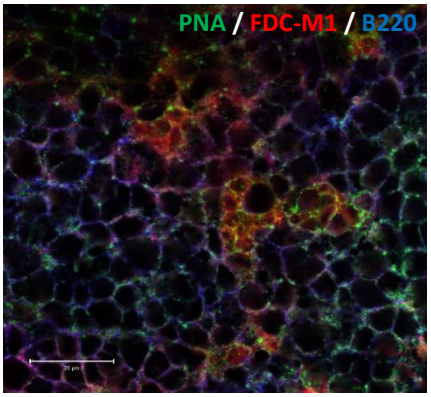

PNA / FDC-M1 / B220

Figure 3.6. Presence of CD4 T cells and FDC network within GC B cells in spleen sections of BL6 recipients of bm12 heart grafts at week 7 post transplantation

a. Staining was performed with anti-PNA Ab (upper left) and anti CD4 Ab (upper right) and anti-B220 Ab (lower left). Co-localization of B220, PNA and CD4 (lower right) highlighting the presence of CD4 T cells in GCs highlighted with a white box in lower right panel (x20). The area of co-localisation of CD4 T cells with B cells and GC was zoomed in to show the close proximity of CD4 T cells (red) with GC (green) B cells (Blue) as shown in figure on the right with a white circle.
b. Presence of FDC network within GCs at 20x magnification. Staining was performed with anti-PNA Ab (upper left) and anti FDC-M1 (upper right) and anti-B220 Ab (lower left). Co-localization of B220, PNA and FDC (lower right) highlighting the presence of FDC network in GCs.
c. This FDC network was further examined at 63x magnification where the network was better appreciated. Staining was performed with anti-PNA Ab (upper left) and anti FDC (upper right) and anti-B220 Ab (lower left). Co-localization of B220, PNA and FDC (lower right) highlighting the presence of FDC network in GCs.
d. Merged figure of PNA, FDC and B200 showing close proximity of FDCs with GC B cells (x63), scale bar is 20µm.

Having confirmed GC response in this model at week 7 post transplantation, the next question was when they start and how long they last. GC responses were quantified in recipient spleen at days 7, 15, 50 and 100 after transplantation, by staining with germinal centre markers (PNA and or GL 7) and B cell markers. There were no germinal centres evident at day 7. However, GCs were first observed at day 15, with more than 30% of the B cell follicles exhibiting a GC phenotype. By day 50, more than 50% of the B cell follicle stained positively for GC markers, with a GC response still present 100 days after transplant (figure 3.7).

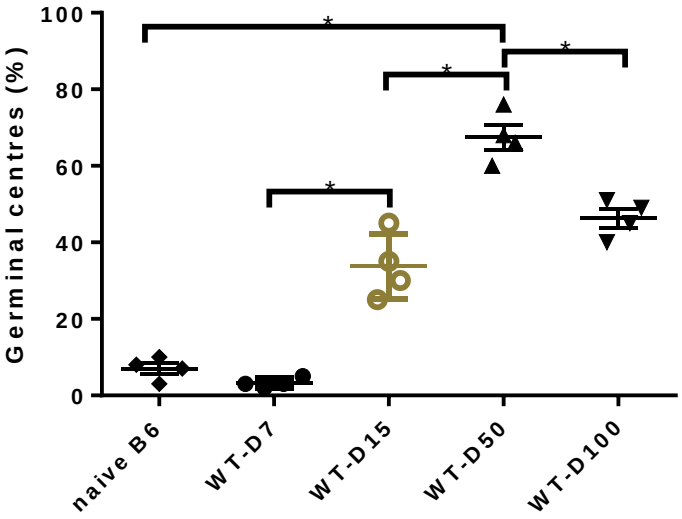


Figure 3.7. Quantification of GCs at various time points in BL6 recipients of bm12 allografts

Recipient splenic sections showing the appearance of GCs at day 15, and their presence till day 100 post transplantation.

Data represent mean and SD of n = 4 mice per group, **P* < 0.05, (Mann-Whitney test)**.**

3.3.4. Identification of T_{FH} signature markers of CD4 T cells

Although other subsets of follicular helper cells have been described, the CD4 T_{FH} cell is still considered fundamental for GC responses against conventional T-dependent protein antigen (Crotty, 2011; Crotty, 2014; Crotty, 2015; Qi, 2012; Vinuesa and Cyster, 2011). I characterised this subset in my model by staining recipient splenocytes with anti CXCR5 and anti PD1 antibodies as has been described in the literature (Crotty, 2011; Yu and Vinuesa, 2010; Yusuf et al., 2010). Staining was carried out as described in methods (section 2.2.3)

On flow cytometric analysis, 3.1±0.9 % of the live activated CD4 T cells acquired the T_{FH} cell phenotype as characterised by CXCR5hi and PD1hi, when compared to naïve BL6 CD4 T cells (0.4±.1%) (figure 3.8). It is important to mention here that flow pattern did not reveal a distinct population of T_{FH} CD4 T cell, but rather a shoulder and so it is difficult to describe exactly.

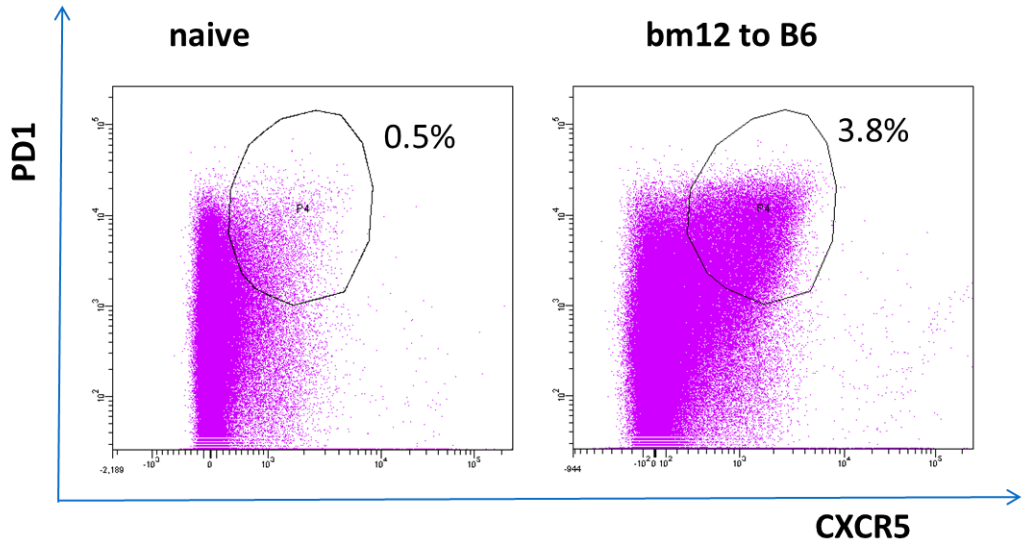


Figure 3.8. Identification of follicular T helper cells using flow cytometry at day 15 in splenocytes of BL6 recipients of bm12 heart grafts

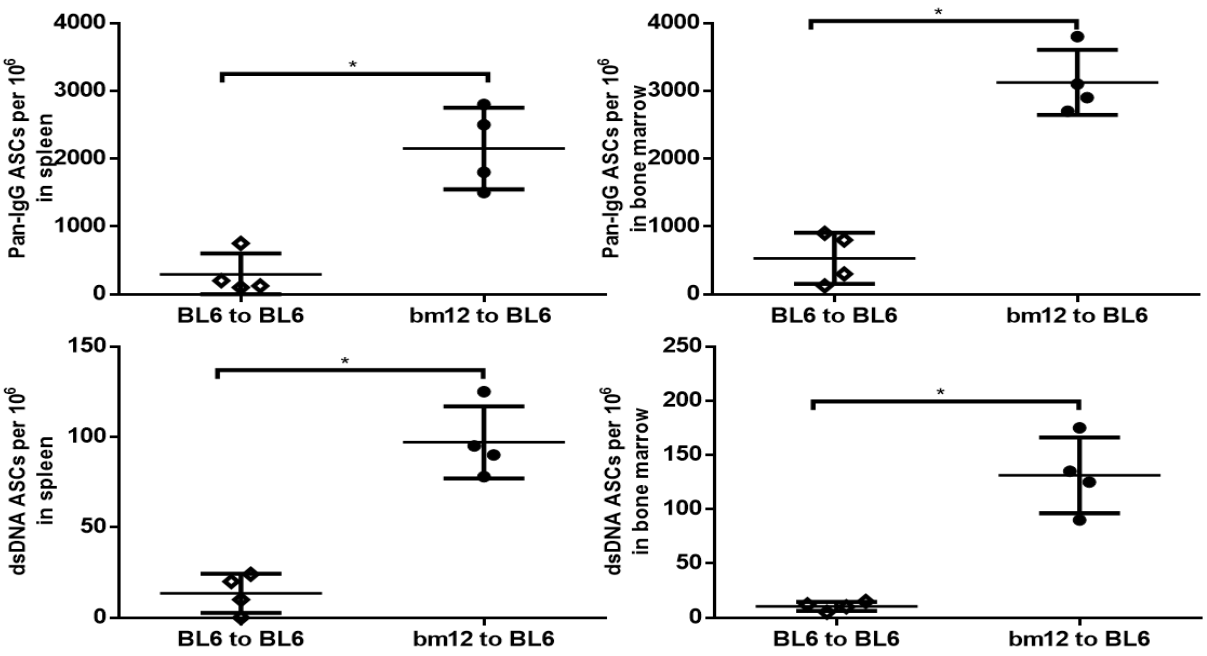
The live CD44hi CD4 T cell population was selected and T_{FH} cells were identified on the basis of dual staining with CXCR5hi and PD1hi. Naive unchallenged BL6 CD4 T cells are shown as a control T_{FH} cell population (n=3).

3.3.5. Confirmation of the longevity of humoral response by ELISPOT

Because circulating autoantibodies are detected to three months following transplantation in this model, I next addressed whether antibody secreting cells (ASCs) could be detected in recipients' spleen and or bone marrow. ELISPOT assays was performed in BL6 recipients' splenocytes and bone marrow cells at week 7 post transplantation in order to detect pan IgG ASCs and dsDNA specific ASCs, by coating ELISPOT plates with pan IgG and dsDNA autoantigens (see section 2.2.6).

ELISPOT assay showed the presence of significantly higher number of positive spots for ASCs cells for pan IgG and dsDNA antibodies in BL6 recipients of bm12 allografts, both in bone marrow and spleens when compared with the syngeneic controls (figure 3.9). Within the BL6 recipients of bm12 allografts group, there was no difference in the APCs between spleen and bone marrow at week 7 post transplantation.

a.


Pan-IgG ASCs per 10^6 in spleen
4000
3000
2000
1000
0
*
BL6 to BL6
bm12 to BL6
Pan-IgG ASCs per 10^6 in bone marrow
4000
3000
2000
1000
0
*
BL6 to BL6
bm12 to BL6
dsDNA ASCs per 10^6 in spleen
150
100
50
0
*
BL6 to BL6
bm12 to BL6
dsDNA ASCs per 10^6 in bone marrow
250
200
150
100
50
0
*
BL6 to BL6
bm12 to BL6

b.

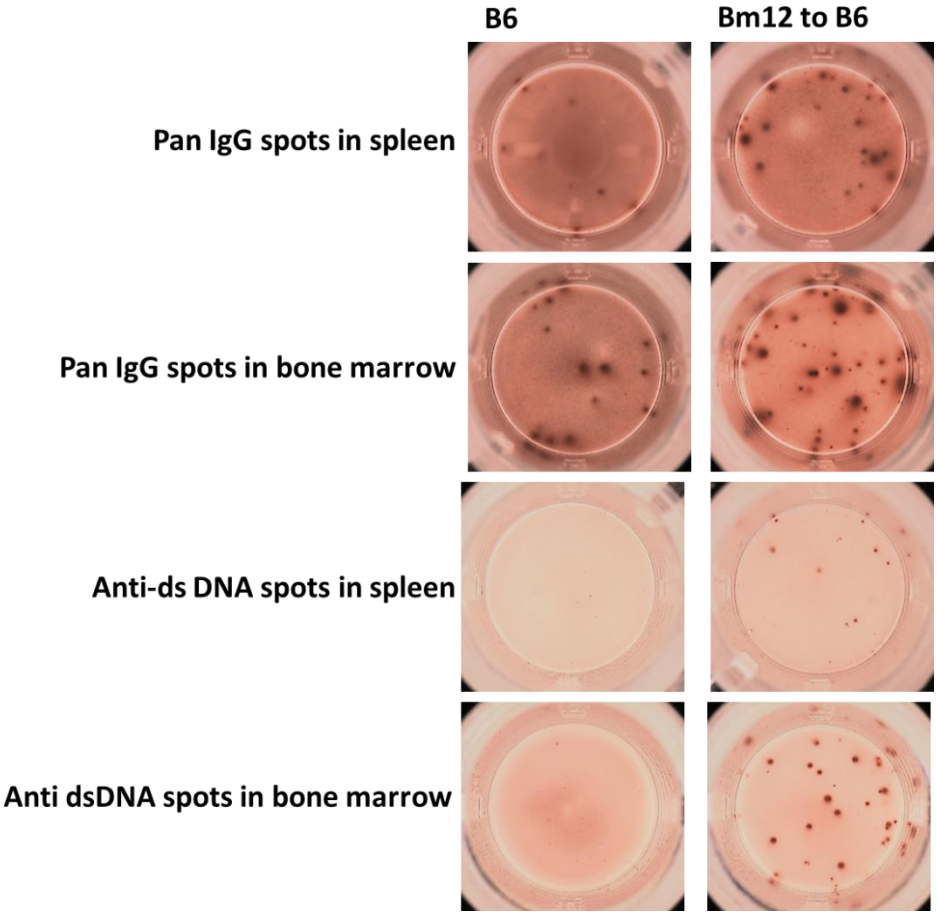

B6
Bm12 to B6
Pan IgG spots in spleen
Pan IgG spots in bone marrow
Anti-ds DNA spots in spleen
Anti dsDNA spots in bone marrow

Figure 3.9. Longevity of autoantibody responses demonstrated by ELISPOT assay

a. Statistically significant number of antibody secreting cell (ASC) spots were demonstrated in the autoantibody producing group (bm12 to BL6) for pan-IgG and anti-dsDNA antibody both in spleen and bone marrow compared to syngenic grafts (n=4). This assay was performed in recipients' splenocytes and bone marrow cells at 7 weeks post transplantation.
b. Representative figure of spots for IgG and dsDNA-antibody secreting cell in naïve BL6 and BL6 recipient of bm12 allograft in spleen and bone marrow at 7 weeks post-transplantation.

Data represent mean and SD of n = 4 mice per group, (Mann-Whitney test)**.**

3.3.6. Is there a diversification of humoral response?

3.3.6.1. Indirect immunofluorescence images from the same animal showing different patterns

Germinal centres underpin clinical humoral autoimmunity and during GC response, different proteins are typically targeted. This raises the potential that the GC response and specifically SHM may lead to epitope diversification and development of response against new target autoantigens.

The images of the hep-2 indirect immunofluorescence slides from the BL6 recipients of bm12 allografts revealed different patterns of staining within the same animal over time following transplantation (see figure 3.10), suggesting that a variety of nuclear and cytoplasmic autoantigens can act as targets for the humoral autoimmune response.

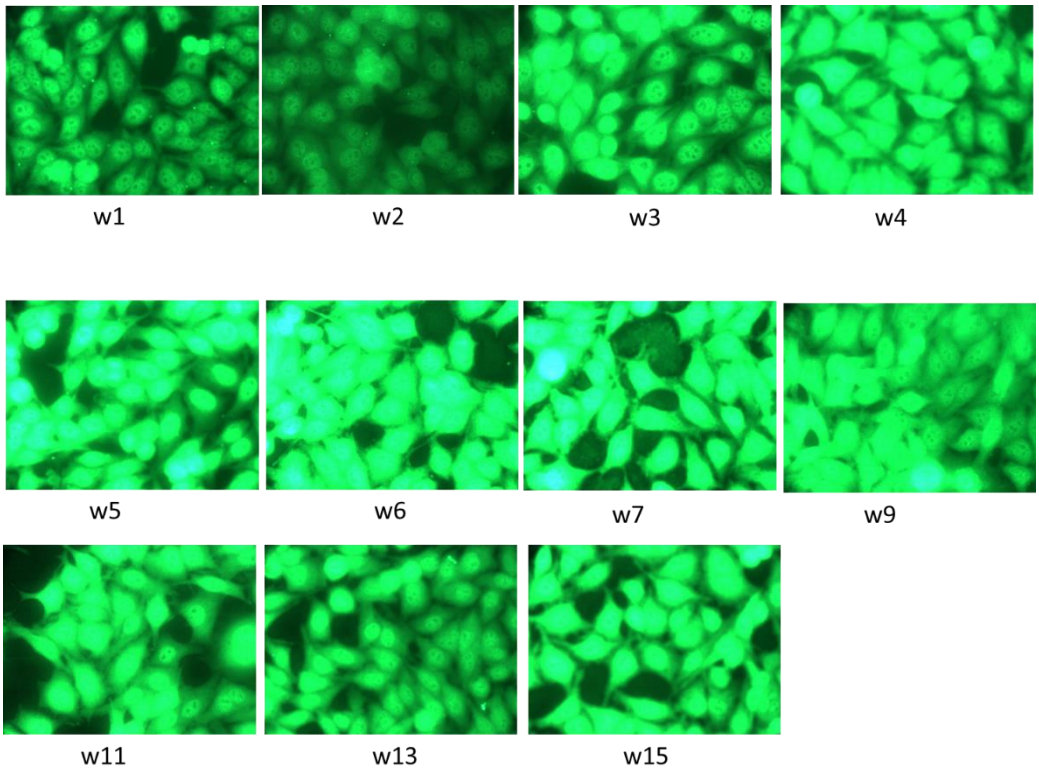


Figure 3.10.Identification of various patterns of autoantibodies in BL6 recipient of bm12 heart graft on hep-2 indirect immunofluorescence

Various patterns from week (wk) 1 to wk 15, seen on hep-2 indirect immunofluorescence demonstrating the presence of various types of anti-nuclear and cytoplasmic autoantibodies. All pictures are taken at x20 magnification.

3.3.6.2. Development of late anti-vimentin on ELISA

Diversification of the humoral response was assessed by examining the development of anti-vimentin autoantibody in the recipients at early and late time points. Vimentin autoantibody was selected as a surrogate marker for diversification of humoral response as it has been shown contributing to chronic rejection in experimental rodent models and in clinical transplantation (Mahesh et al., 2007; Rose, 2013; Rose and Smith, 2009).

Anti-vimentin autoantibody was detected by performing ELISA assay on recipient sera at week 7 and week 15. ELISA assay was performed as described in section 2.2.5.3.

Anti-vimentin autoantibody ELISA of recipient sera demonstrated an increase in anti-vimentin at a late time point compared to early time point as shown below in figure 3.11. Notably, this autoantibody was detectable by week 15 in approximately half of WT recipients of bm12 heat allografts.

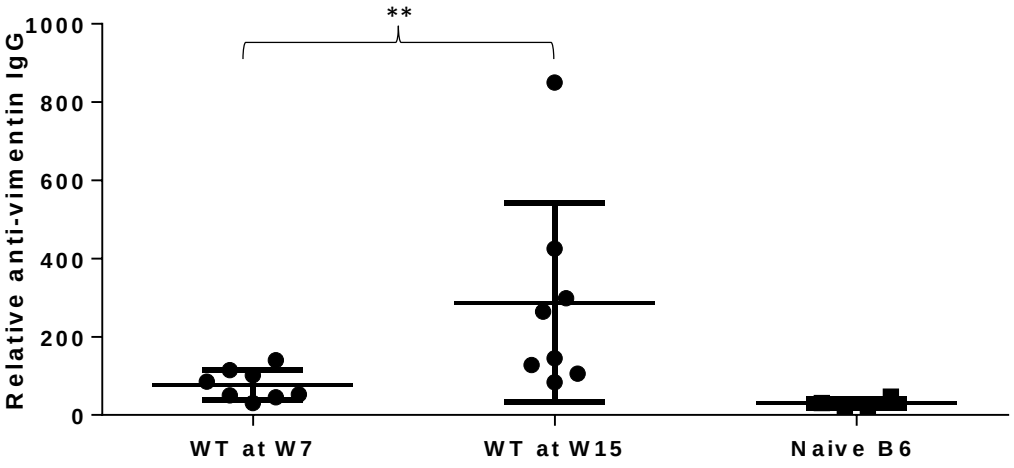


Figure 3.11. Diversification of humoral responses as demonstrated by development of anti-vimentin autoantibody at week 15 post transplantation

Anti-vimentin ELISA demonstrating the levels of anti-vimentin IgG antibody at early (week 7) and late time points (week 15) (p=0.007), following transplantation of bm12 heart grafts into WT BL6.

Data represent mean and SD of n = 5-8 mice per group, $^{**}P < 0.01$, Mann-Whitney test was used.

3.4. Summary

In summary, we conclude from this chapter that,

1. Long-lasting germinal centre responses are observed in recipients of chronically-rejecting heart allografts.
2. Germinal centre humoral immunity was associated with deposition of antibody producing cells in recipient spleen and bone-marrow.
3. T_{FH} CD4 T cells were identified on the basis of their homing to the secondary follicle and by expression of characteristic phenotypic markers CXCR5 and PD1.
4. The humoral autoimmune response may diversify at late time point to incorporate reactivity against vimentin.

3.5. Discussion

Germinal centre responses against conventional protein antigens have been extensively studied in the last few years, but have not so far been described in transplantation. In this chapter, GC responses were characterised by immunofluorescence staining and flow cytometry staining in a model of chronic rejection, in which the humoral autoimmune response is dependent upon graft versus host recognition by passenger donor CD4 T cells (Win et al., 2009). On immunofluorescence staining, GCs were identified by GL7 and / or PNA positive B cell follicles in recipient spleen sections following allograft transplantation and homing of CD4 T cells within these germinal centres (figure 3.4, 3.5 and 3.6). On flow cytometry staining, a subset of CD4 T cells was identified possessing dual positivity for CXCR5hi and PD1hi surface markers (figure 3.8), presumably representing the follicular helper CD4 T cell subset. Furthermore, deposition of LLPC within the bone marrow as shown by ELISPOT assay (figure 3.9) may be representing the long lived nature of humoral autoimmunity in this model.

T-dependent humoral responses are composed classically of two components. The initial extrafollicular component provides early immunity, whereas the germinal centre component provides long lasting durable immunity. In bm12 to BL6 heart allograft model, weak autoantibody responses are detectable early (in the first 2 weeks), which presumably represent an extrafollicular response. After 2 weeks, autoantibody titres rise quickly and plateau at between week 5 and 7, and remain detectable thereafter. These later responses are likely the result of a germinal centre response. The presence of this long lasting antibody in the recipients is similar to transplant recipients who are sensitised or who have experienced antibody mediated rejection several years post transplantation. Despite the fact that in this model, humoral response is directed against self-antigens, there are some similarities with clinical transplantation, in that donor specific alloantibody that develops after transplantation, is associated with poorer transplant outcomes, is also long-lasting, and presumably an output of a germinal centre response. Due to these similarities, a deeper understanding of transplant associated autoimmunity (TAA) may be helpful in elucidating the underlying mechanisms responsible for the development of allograft vasculopathy, and may inform the development of approaches that prolong allograft survival.

GCs were characterised on immunofluorescence staining by PNA positivity and or GL 7 positivity of the B cells (Butcher et al., 1982; Coico et al., 1983; Rose et al., 1980). The critical step in the development of these long lasting and class switched (CS) GC autoantibodies is the help provided to B cells by T_{FH} cells. T_{FH} cells are defined by their homing into the B cell follicle, coupled with surface markers such as CXCR5, PD1 and ICOS (Vinuesa et al., 2005; Yu and Vinuesa, 2010). Consistent with this, I could identify CD4 T cells co-localising within the GC areas on microscopy (figure 3.6), and flow cytometric analysis revealed a subset of CD4 T cells with the signature $CXCR5^{hi}$, $PD1^{hi}$ markers (figure 3.8).

Timing of development of the GCs may be different for different immunogenic challenges. For a conventional immunogenic challenge, the GCs have been described to appear as early as day 7 post immunisation (Blink et al., 2005; Garside et al., 1998), and then regress between week 3 to week 14 (Bachmann et al., 1996; Jacob et al., 1991; Kelsoe, 1996; Liu et al., 1991). However, in this model of chronic rejection, the development of GCs took almost 2 weeks to appear as seen with IF (figure 3.7). But, it may be possible that the IF may not be robust enough to detect the development of GCs during week one.

Germinal centres are long lasting in this model and can be seen up to three months post transplantation (figure 3.7). The long lasting presence of GCs may be due to the continuous presence of antigens in the form of heart graft (Bachmann et al., 1996) or due to the SHM which may lead to epitope diversification and development of response against new target autoantigens (MacLennan, 1994). In support, development of anti-vimentin autoantibody at a late time point has been seen in this model (figure 3.11). Furthermore, it may be possible that long lived GCs are due to re-utilisation of autoreactive GCs by heterologous antigens as the activated B cells will prefer to enter into an existing GC where the existing structure already persist (Bergqvist et al., 2013; Schwickert et al., 2009). However, it is not possible to tease out the re-utilisation hypothesis in this model as polyclonal B cells are responding to polyclonal CD4 T cells.

Each individual GC is thought to be seeded by a very small number of oligoclonal B cells that then undergo massive expansion (Faro and Or-Guil, 2013). B cells acquire antigen from FDCs and then process and present that antigen to T helper cells at the T-B cell border. Prolonged interaction with those CD4 T cells results in differentiation into the T_{FH} cell subset and in return the B cells receive survival signal from T_{FH} cells to form GCs (Garside et al., 1998;

Linterman et al., 2012; MacLennan, 1994). Different GCs will have different antibodies outputs and memory B cells depending upon the type of antigens they were challenged and at different time points different antibodies can be seen. Like in SLE, a wider spectrum of autoantibodies is seen and their titres vary with time which may correspond to the disease activity as well. In my work, as the GCs are autoreactive, hence each of the GC might be producing different autoantibody. Some of them may be specific to ssDNA autoantigen, some may be to dsDNA and others may be against chromatin or vimentin. Different types of autoantibodies are observed as shown by different patterns of autoantibodies on indirect immunofluorescence hep-2 analysis (figure 3.10) and development of late anti vimentin on ELISA (figure 3.11). On indirect immunofluorescence, the different patterns of autoantibodies were observed even within the same animal at different time points (figure 3.10). These included various types of anti-nuclear autoantibodies and cytoplasmic autoantibodies. However, the fluorescence intensity of the cytoplasmic autoantibodies was very high and it was difficult to identify a definite pattern of cytoplasmic autoantibodies on analysis. It will be interesting to examine it further by diluting the recipient sera to characterise definite patterns of autoantibodies which may be helpful in determining the contribution of various types of autoantibodies to chronic rejection. The development of this diversified humoral response raised two questions: whether the GCs contribute to diversification of humoral responses and whether this diversification contributes to graft failure. Both these questions are addressed in later chapters.

One of the outcomes of GCs is deposition of LLPC in the bone marrow niche, which can produce antibody for the life time of the individual. The rate limiting factor for survival of plasma cells is their bone marrow niche. If a niche is destroyed or plasma cells leave its niche, it will soon die (Manz et al., 2002). Spleen can also be a source of antibody production (Slifka et al., 1995), but whether it can retain LLPCs (Sze et al., 2000), as happens in the bone marrow, is contentious. Hoyer at al (Hoyer et al., 2004) has shown in a murine model of SLE (NZB/W) that 40% of the ASCs from the spleen were non-dividing and long lived plasma cells. In my work, comparable number of ASCs specific for IgG and dsDNA were seen both in bone marrow and spleen on ELISPOT assay (figure 3.9). However, these ASCs needs to be further characterised to establish whether there is a difference in the terminally differentiated non-dividing plasma cells between spleen and bone marrow.

The present results suggest that long-lasting germinal centre responses are observed in recipients of chronically-rejecting heart allografts which needs further exploration to address the following questions;

- What is the contribution of donor and recipient CD4 T cells to the development of these GCs?
- Are GCs central to diversification of humoral responses?
- Do GC autoantibody responses contribute to progression of allograft vasculopathy?

Chapter 4

Host T_{FH} cell is required for the development of long term germinal centre autoantibody responses

4.1. Introduction

In the previous chapter, I have shown that humoral autoimmunity triggered by heart transplantation is long lasting, with strong titres maintained three months after transplantation. Recipient splenic sections demonstrated the presence of GCs on confocal immunofluorescence microscopy. Within these GCs, CD4 T cells were also identified, in keeping with a population of T_{FH} cells that are providing essential help for maintenance of the GC (Crotty, 2015; Linterman et al., 2009; Qi, 2012). Abnormalities in antigen availability, T cell help or the threshold at which B cells respond to these stimuli in germinal centres can result in the development of long lasting autoimmunity in the recipients (Linterman et al., 2009; Vinuesa et al., 2009). In Linterman et al work, this autoimmunity was triggered due to Roquin-mediated dysregulation of T cells which resulted in aberrant expansion of T_{FH} subset and development of spontaneous autoreactive GCs (Linterman et al., 2009). These findings raised important and yet unanswered questions with respect to my work. Firstly, whether passenger donor CD4 T cells within a heart graft trigger a GC autoantibody response and if so, whether the autoreactive T_{FH} cells are of donor origin or recipient origin or both? If the T_{FH} cells are donor derived, this indicates their survival in an allogenic host. In contrast, if T_{FH} cells are recipient-derived, this suggests that GVH responses trigger secondary activation of an autoreactive recipient CD4 T cell population, possibly through the ability of activated autoreactive B cells to act as professional APCs and present peptide autoantigens for self-restricted recognition by naïve autoreactive recipient CD4 T cells. The latter concept – that one 'dysfunctional' population of helper CD4 T cells may trigger humoral autoimmunity that is then sustained by T_{FH} differentiation of a second population of CD4 T cells, has not been previously described and would have important implications for how autoimmune disease manifests. Investigation of the relative roles of donor and recipient CD4 T cells in providing T_{FH} cell function in our transplant model may therefore have wider relevance for autoimmune disease in general.

Aside from durable production of autoantibody, long-lasting autoreactive GCs may also contribute to autoimmune disease progression by promoting diversification of the B cell response to incorporate different target autoantigens, some of which may be particularly pathogenic. This process is termed as T and B cell epitope diversification (Mitchison, 1971; Rajewsky et al., 1969). Epitope diversification has been described differently by investigators

based on their work (Fatenejad et al., 1993; Mamula, 1998; Mamula and Janeway, 1993; Mamula et al., 1992; Shlomchik et al., 2001; Singh and Hahn, 1998). Mamula and Janeway suggested the role of B cells as an efficient antigen presenting cell for various components of antigen and thus recruiting naïve CD4 T cell to provide help to B cells for development of antibodies (Mamula and Janeway, 1993; Mamula et al., 1992). Their experimental work demonstrated that immune responses are initiated by priming of CD4 T cells by immune-dominant epitopes presented by APCs like DCs. The primed CD4 T cells then activate the antigen specific B cells which internalise the antigen more efficiently and in high concentrations and become competent in activating more naïve CD4 T cells for progression of this autoimmune phenomena. They have shown this by eliciting autoimmune responses to mouse autoantigen cytochrome c and ribonucleoproteins. Priming with one immunogenic peptide of one autoantigen was able to generate CD4 T cells responses to other peptides within the same protein like cytochrome c (Mamula et al., 1992).

On the other hand, Singh et al (Singh and Hahn, 1998) has described this as a reciprocal T-B determinant spreading where the induction of first T cell by peptides from an autoantibody molecule could lead to help provided to a variety of B cells displaying a cross-reactive version of the original determinant of the autoantibody. This response spreads in this way by reciprocal T-B stimulation until large cohorts of T and B cells have been expanded. This whole concept is shown below by copying figure form Singh et al paper (Singh and Hahn, 1998).

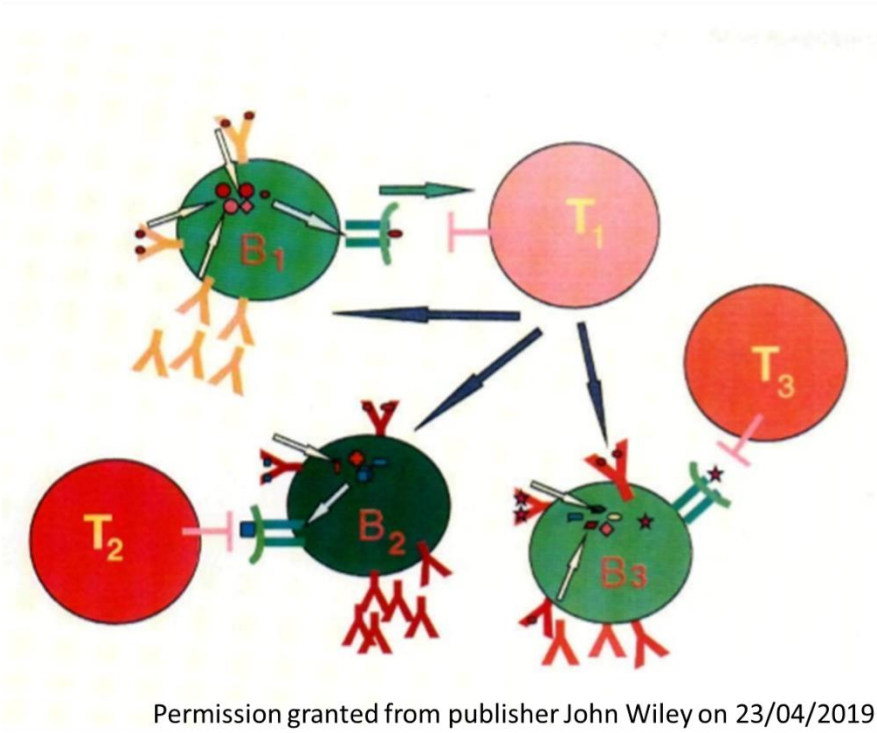


Fig. 1. Reciprocal T–B-cell diversification: peptides derived from Ig activate T-cell help for autoantibody production. A B cell (B_1, green) is shown processing its own surface Ig molecules (Y shaped) into peptides. Some of the peptides (red) are then presented by the MHC class II molecules (shown as a large Roman numeral II on the B-cell surface) to the TCR (shown on the surface of T-cell, T_1, as a large T). This engagement then results in activation of T-cell help (indicated by the green arrow), which in turn activates the B cell to secrete more of the antibody displayed on its surface. T_1 also gives help to other B cells (B_2 and B_3), which share the original determinant (red) on Ig secreted by the first B cell (B_1). B_2 and B_3 in turn process the same or different determinants (shown as blue rectangle or red star), and activate T_2 and T_3 respectively. This mechanism could contribute to sustained production of not only the original autoantibody but different antibodies that share T-cell determinants in their V regions.

Permission granted from publisher John Wiley on 23/04/2019 for this figure (Singh and Hahn, 1998)

However, Shlomchik et al suggested (Shlomchik et al., 2001) a positive feedback mechanism between T-B cell interaction and new autoantigen release following any organ damage in autoimmune prone conditions. Once T cells are primed by DCs, they can activate the genetically prone autoimmune B cells. These autoantigen-stimulated B cells undergo hypermutations and affinity maturation resulting in the development of pathological autoan tibodies. These pathological autoantibodies damage the organs and result in the release of further self-antigens which are again taken up by specific antigen presenting B cells in a second round of T cell activation. Autoreactive humoral autoimmune responses are typically long-lasting and thus potentially a facet of a GC reaction, but whether this diversification is exclusively a consequence of the GC has not been clarified.

We have shown the development of dsDNA autoantibodies in this model previously (Win et al., 2009) and the late development of anti-vimentin autoantibody in the previous chapter (Figure 3.10). The model therefore provides the opportunity to investigate GC-mediated epitope diversification, by using the development of anti-vimentin autoantibody as a marker of that diversification.

4.2. Aims

The aims of this chapter are:

1. To determine the role of donor and recipient CD4 T cells in the development of GC autoantibody responses.
2. To investigate the contribution of germinal centres to diversification of humoral responses.
3. To examine whether a monoclonal population of CD4 T cells is sufficient to drive diversification of humoral response, or whether additional T cell subsets are required.

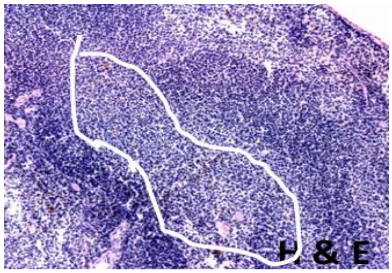
4.3. Results

4.3.1. Replication of the same results with adoptive transfer of cells as those observed with murine allografts

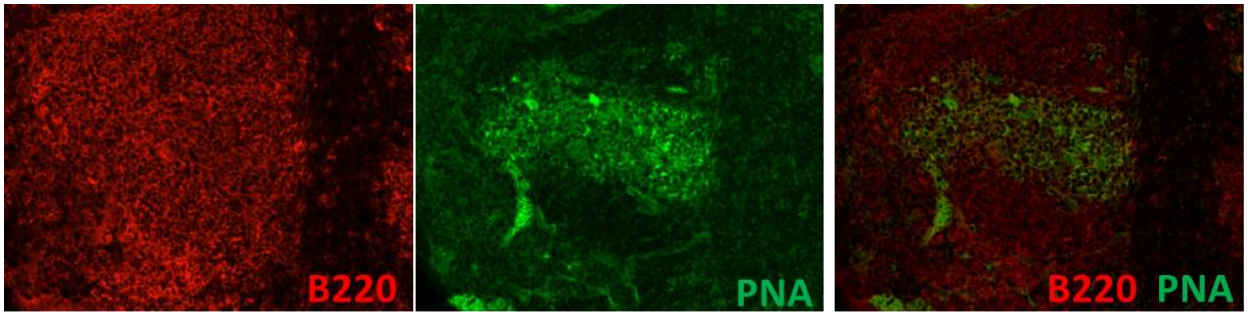
Adoptive transfer of column purified bm12 CD4 T cells was carried out into BL6 recipients. CD4 T cells were purified as described in section 2.2.1.5. Recipient's splenic sections were stained with anti-B220 and anti-GL 7 and or anti-B220 and anti-PNA to identify and quantify GCs at 7 weeks after adoptive transfer. Recipients' sera were examined for autoantibodies by hep-2 indirect immunofluorescence. Of note, in all adoptive transfer experiments, $2x10^6$ purified CD4 T cells were adoptively transferred into recipients unless stated otherwise.

On staining of recipient splenic sections with GC markers, more than 60% of the follicles differentiated into GCs (figure 4.1). This demonstrated that adoptive transfer of bm12 CD4 T cells replicated the results as those observed with the bm12 allografts.

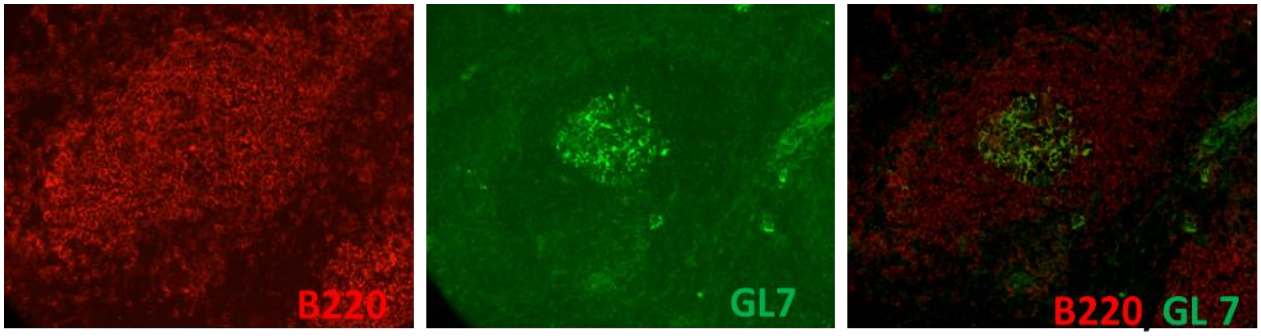
a.


H & E

b.


B220
PNA
B220 PNA

c.


B220
GL7
B220 GL 7

d.

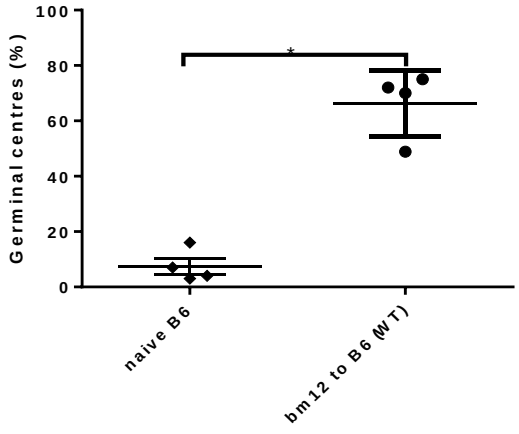

Germinal centres (%)
100
80
60
40
20
0
*
naive B6
bm12 to B6 (WT)

Figure 4.1. Development of germinal centres in BL6 recipients of bm12 CD4 T cells at week 7 after adoptive transfer.

a. A representative image of dark zone and light zone in secondary splenic B cell follicle on haematoxylin and eosin staining of splenic sections at week 7 after adoptive transfer of bm12 CD4 T cells into BL6.
b. Immunofluorescence staining of spleen sections showing presence of germinal centres in BL6 recipient of bm12 CD4 T cells. Staining was performed with anti-B220 Ab (left) and anti PNA (middle) and co-localised B220 and PNA staining (right). All the pictures were taken at 20x magnification
c. Immunofluorescence staining of spleen sections showing presence of germinal centres in BL6 recipient of bm12 CD4 T cells. Staining was performed with anti-B220 Ab (left) and anti GL 7 (middle) and co-localised B220 and GL 7 staining (right). All the pictures were taken at 20x magnification.
d. Quantification of germinal centres in BL6 recipients of bm12 CD4 T cells or naïve BL6 at week 7 post adoptive transfer. This showed that more than 60% of the primary follicles differentiated into secondary follicles or germinal centres as demonstrated by PNA^+ and / GL 7^+ B220 follicles in the WT BL6 recipients of bm12 CD4 T cells compared to naïve BL6 (n=4) (p=0.01).

Consistent with this, there was development of long lasting autoimmunity in the recipients, as demonstrated by presence of long lasting IgG autoantibodies in recipient's sera (figure 4.2).

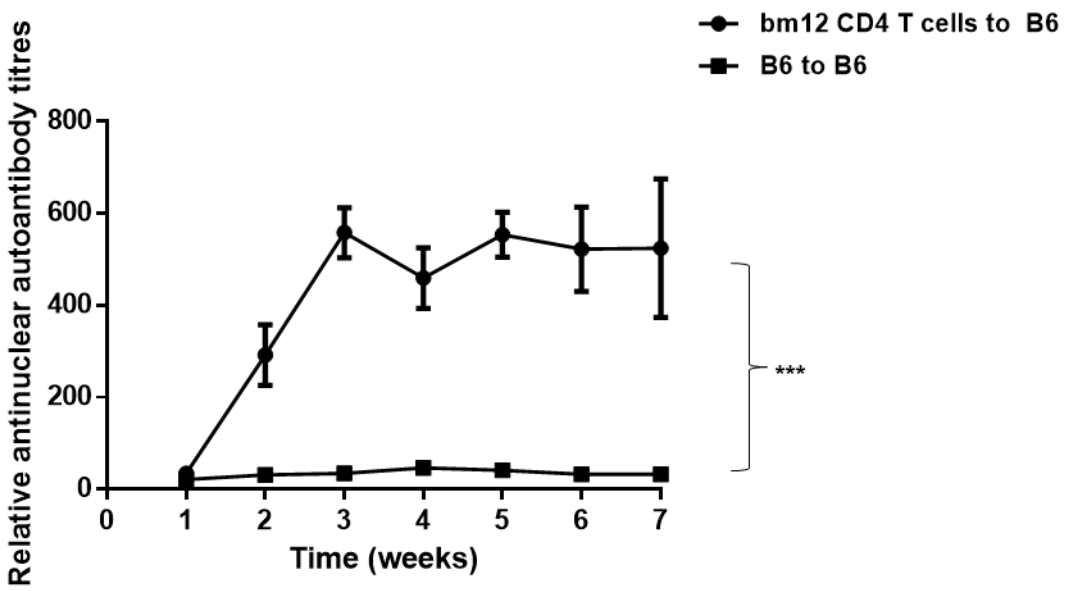
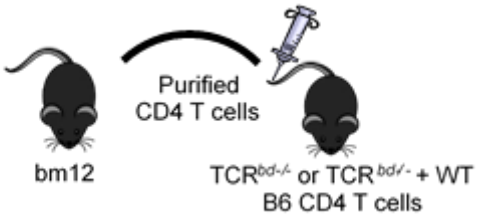


Figure 4.2. Development of long lasting autoantibodies in BL6 recipients of bm12 CD4 T cells

Relative anti-nuclear autoantibody levels as measured by Hep-2 indirect immunofluorescence in BL6 recipients sera demonstrating development of long lasting autoantibodies in BL6 recipients of bm12 CD4 T cells compared to BL6 recipients who received syngenic BL6 CD4 T cells (p=<0.0001, Two way ANNOVA). Data represent mean and SD of n = 4 mice per group.

Having established that adoptive transfer of bm12 CD4 T cells generated long lasting GC humoral autoimmunity in wild-type BL6 recipients, I next sought to investigate the contribution of recipient CD4 T cells to the response. Thus, T cell deficient BL6 recipients ($TCR^{bd-/-}$) received either column purified donor bm12 CD4 T cells alone ($2x10^6$ cells), or donor bm12 ($2x10^6$ cells) and WT BL6 recipient ($4x10^6$ cells) CD4 T cells simultaneously (figure 4.3).



	Donor bm12 CD4 T cells	Recipient B6 CD4 T cells
Group 1	+	–
Group 2	+	– (+WT B6)

Figure 4.3. CD4 T cells were manipulated in the BL6 recipients, showing two groups, group 1-without recipient CD4 by using TCR$^{bd-/-}$ transgenic animals and group 2- T cell deficient recipient with both donor and recipient CD4 T cells.

Before proceeding to use TCR$^{bd-/-}$ mice as a recipient for these adoptive transfer experiments, I examined the follicular architecture of B cells in unmanipulated mice from this strain. Follicular architecture was compared between naive TCR$^{bd-/-}$, and TCR$^{bd-/-}$ mice reconstituted with bm12 or BL6 CD4 T cells. Spleens were harvested from naïve TCR$^{bd-/-}$ mice and challenged animals at week 7 after adoptive transfer of purified bm12 or BL6 CD4 T cells or bm12 plus BL6 CD4 T cells simultaneously. On haematoxylin staining of cryo-sections, naïve TCR$^{bd-/-}$ mice and TCR$^{bd-/-}$ mice reconstituted with either bm12 or BL6 CD4 T cells did not demonstrate any secondary follicular pattern. However, simultaneous reconstitution of TCR$^{bd-/-}$ with both bm12 and BL6 CD4 T cells restored secondary follicular architecture as we observe in WT BL6 (figure 4.4).

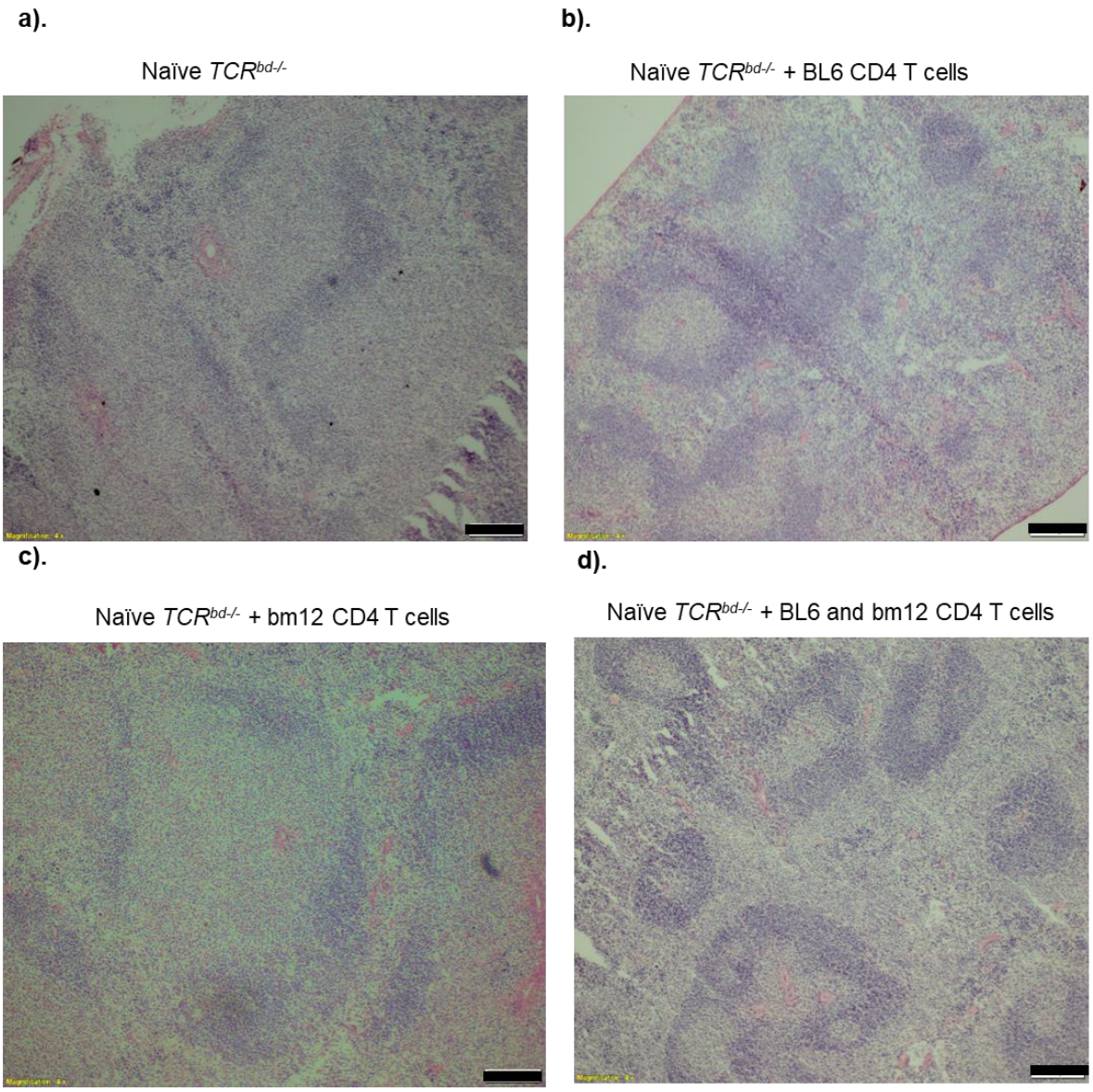


Figure 4.4. Low-power photomicrographs of H&E stained cryostat splenic sections of: non-reconstituted or reconstituted T cell deficient TCR$^{bd-/-}$ mouse

(a); naïve Tcrbd-/- mouse, (b); week 7 after reconstitution with purified BL6 CD4 T cells ($2x10^6$ cells) (c), Seven weeks after reconstitution with purified bm12 CD4 T cells ($2x10^6$ cells) (d), seven weeks after reconstitution with purified BL6 CD4 T cells ($2x10^6$ cells) and simultaneous challenge with purified bm12 CD4 T cells ($2x10^6$ cells). Classical B cell architecture is readily evident in reconstituted mice, with characteristic light and dark zone secondary germinal centre follicles observed in mice simultaneously challenged with purified bm12 CD4 T cells (d). Scale bars 200µm.

4.3.2. Both donor and recipient CD4 T cells are essential for the development of germinal centres

In order to determine the role of recipient CD4 T cells, bm12 CD4 T cells were adoptively transferred into $TCR^{bd-/-}$ (group 1). Recipients' sera and spleen sections were assessed for the development of autoantibody response and GC response respectively.

Hep-2 indirect immunofluorescence demonstrated that the autoantibodies were short-lasting compared to WT responses. Examination of recipient splenic sections with haematoxylin and eosin staining showed only primary follicles that lacked characteristic dark and light zone GC differentiation. Consistent with this, immunofluorescence staining confirmed the absence of a GC response, in that splenic sections did not stain with PNA and / or GL7 staining (figure 4.5).

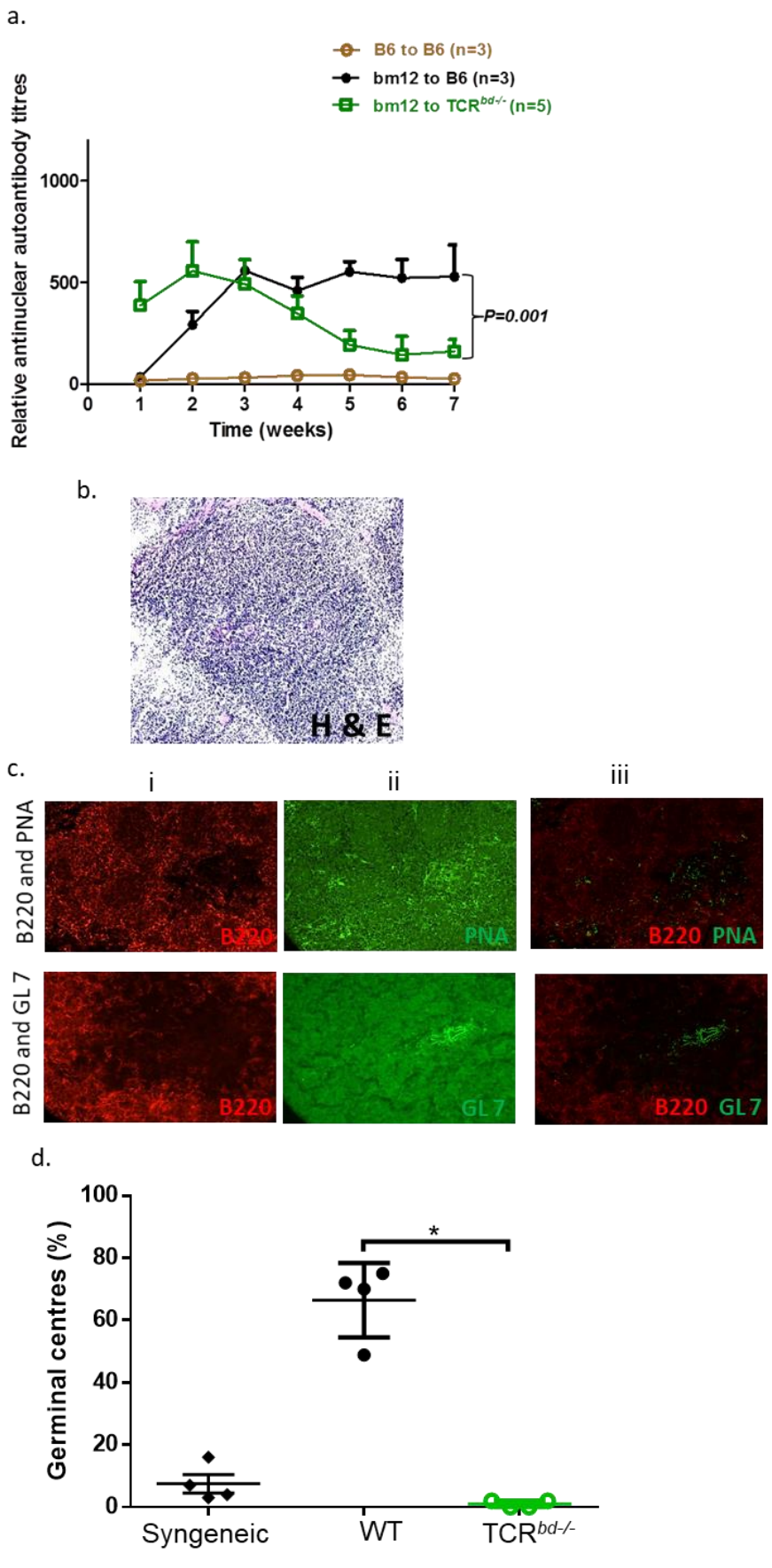


Figure 4.5. Adoptive transfer of bm12 CD4 T cells into TCR$^{bd-/-}$ recipients resulted in the development of short lasting autoantibody and recipient splenic section revealed no germinal centres at 7 weeks post adoptive transfer. All pictures are taken at x20m magnification.

a. Autoantibody levels showing short lasting autoantibodies in TCR$^{bd-/-}$ recipients.

b. H and E staining showing the primary follicles only.

c. Immunofluorescence staining of splenic sections 7 weeks after adoptive transfer of bm12 CD4 T cells into TCR$^{bd-/-}$ .Staining was performed with anti-B220 Ab (red, i panels), anti PNA (green, upper ii panel) and anti-GL 7 Ab (green, lower ii panel). Co-localization of B220 & PNA (upper iii panel) and B220 & GL 7 (lower iii panel) highlighting absence of GCs in the TCR$^{bd-/-}$ recipients.

d. On quantification of GCs, germinal centres were significantly lower than the WT response.

*p=<0.05, Two way ANOVA for a and Mann Whitney test for d was used.

Because circulating autoantibody response was short lasting and without GC response in TCR$^{bd-/-}$ recipients, I next addressed whether antibody secreting cells (ASC) could be detected in recipients' spleen or bone marrow. ELISPOT assays was performed in BL6 recipients' splenocytes and bone marrow cells at week 7 after adoptive transfer in order to detect pan IgG antibody secreting cells (ASCs) and dsDNA specific ASCs, by coating ELISPOT plates with pan IgG and dsDNA autoantigens (see section 2.2.7.).

Consistent with the short lasting autoantibody response, there were significantly fewer spots in TCR$^{bd-/-}$ recipients for pan IgG and anti-dsDNA, both in bone marrow and spleens compared to WT responses (figure 4.6).

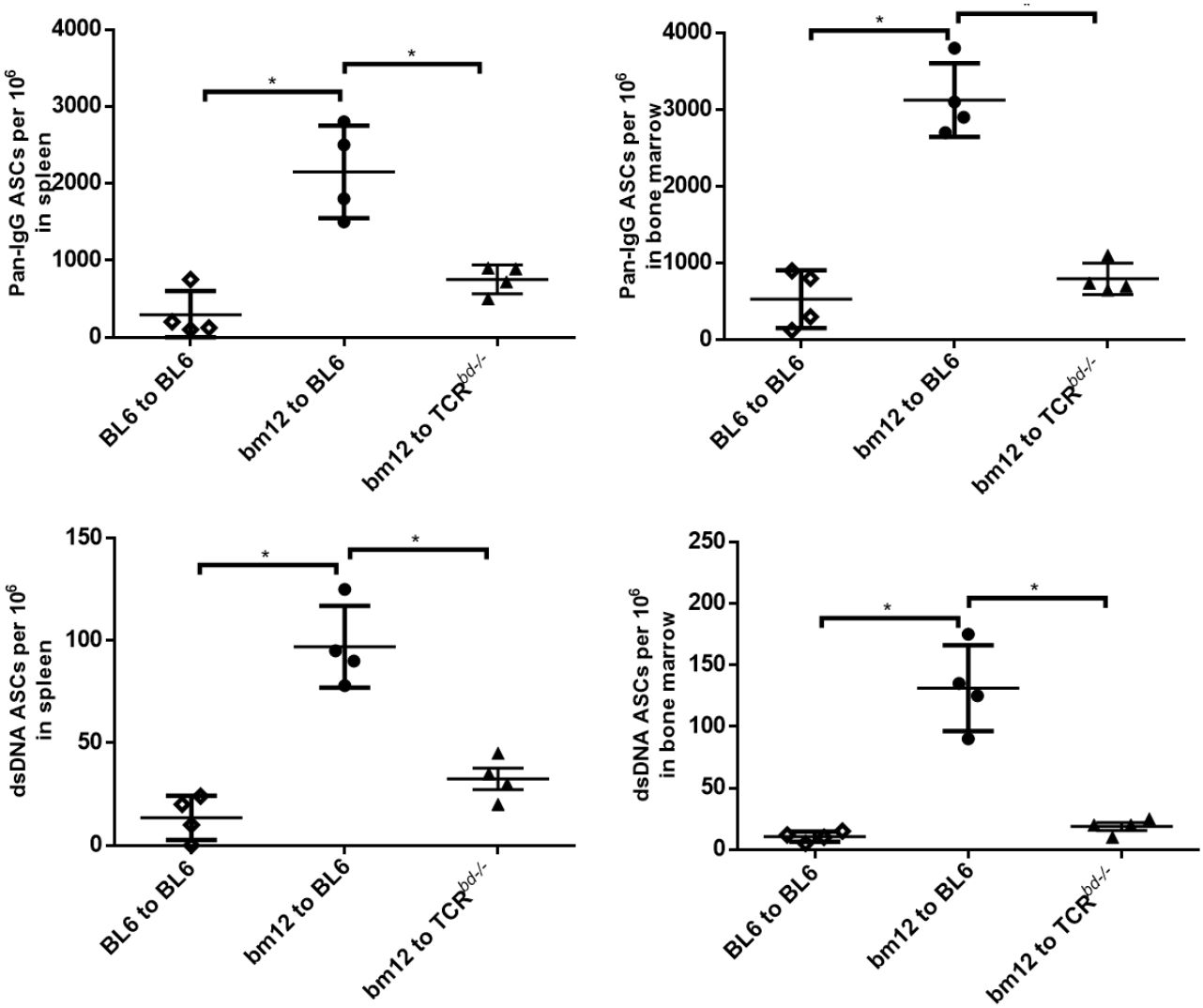


Figure 4.6. ELISPOT assay showing the short lasting humoral response in the absence of GCs in TCR$^{bd-/-}$ recipients following adoptive transfer of bm12 CD4 T cells

Statistically significant lower number of antibody secreting cell spots were demonstrated in TCR$^{bd-/-}$ recipients of bm12 CD4 T cells compared to WT response for pan-IgG and anti-dsDNA antibody both in spleen and bone marrow (n=4), (*p=<0.05, Mann Whitney test was used).

T cell deficient mice challenged with bm12 CD4 T cells showed short lasting humoral response, next I sought whether reconstitution of these mice with donor (bm12 CD4 T cells, $2x10^6$ cells) and host (WT BL6 CD4 T cells, $4x10^6$ cells) simultaneously, will restore GC humoral response at week 7 after the adoptive transfer.

Interestingly, reconstitution of $TCR^{bd-/-}$ recipients with both donor and WT host CD4 T cells restored long lasting autoantibody response and a GC response comparable to that observed in similarly challenged WT recipients (figure 4.7).

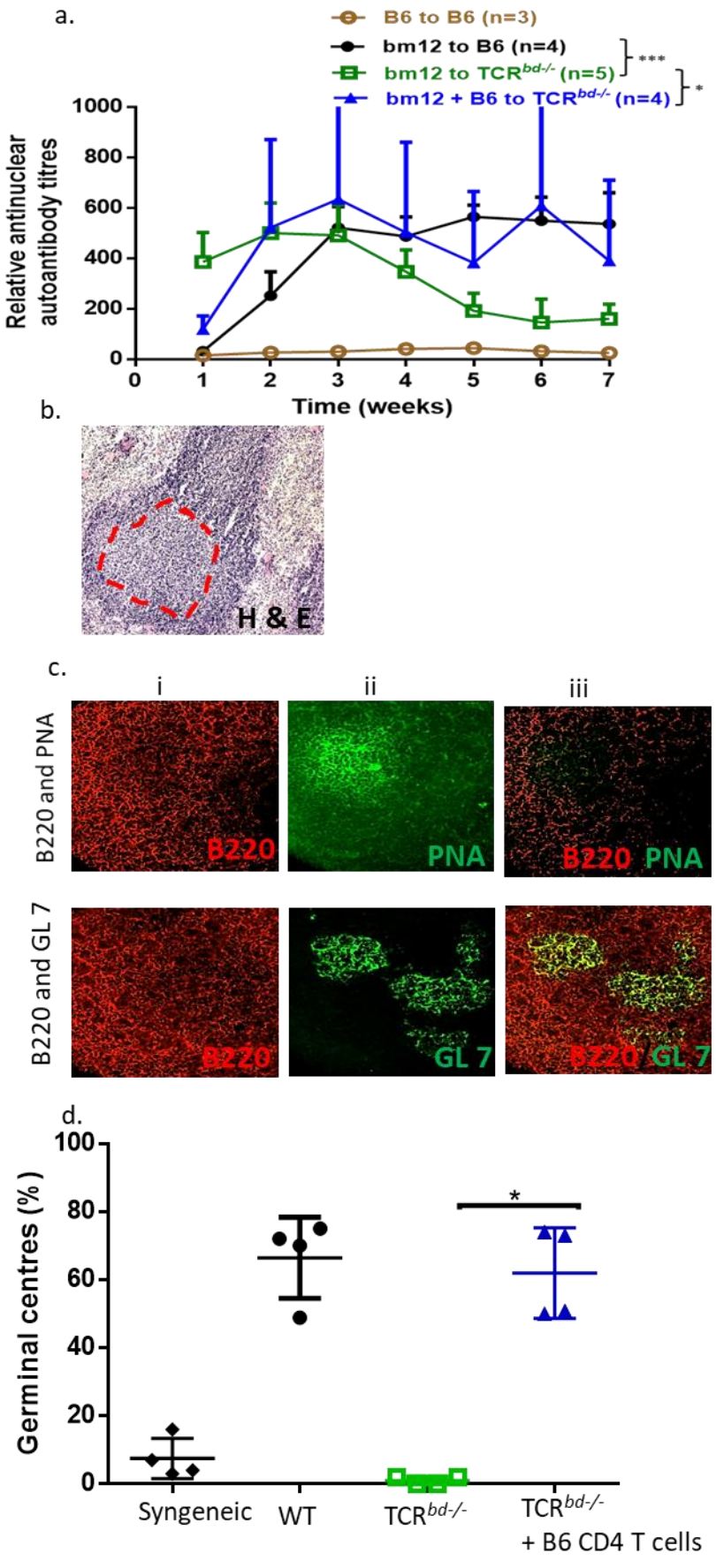


Figure 4.7: Restoration of long lasting GCs on reconstitution of TCR$^{bd-/-}$ with donor and host CD4 T cells.

a. Autoantibody levels in TCR$^{bd-/-}$ recipients reconstituted with donor and recipient CD4 T cells demonstrating long lasting levels of autoantibodies, comparable to WT responses.

b. H and E staining showing the dark zone as demarcated with red dotted line from light zone.

c. Immunofluorescence staining of splenic sections 7 weeks after reconstitution of TCR$^{bd-/-}$ with donor (bm12) and host (BL6) CD4 T cells. Staining was performed with anti-B220 Ab (red, i panels), anti PNA (green, upper ii panel) and anti-GL 7 Ab (green, lower ii panel). Co-localization of B220 & PNA (upper iii panel) and B220 & GL 7 (lower iii panel) highlighting restoration of GCs in the TCR$^{bd-/-}$ recipients reconstituted with WT BL6 CD4 T cells.

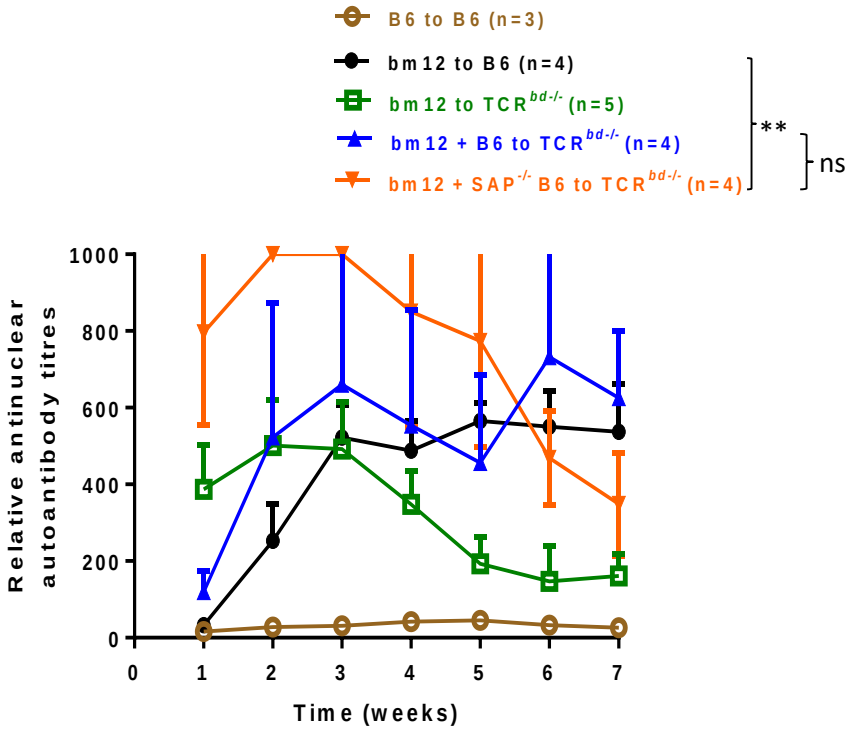
d. Quantification of GCs in splenic sections of the recipients 7 weeks after adoptive transfer of bm12 CD4 T cells into BL6, TCR$^{bd-/-}$ or TCR$^{bd-/-}$ with BL6 CD4 T cells at week 7. (n=4)

*P < 0.05, **P < 0.01 and ***P < 0.001), Two way ANOVA for a and Mann-Whitney test for d.

These experiments thus demonstrate that both donor and recipient CD4 T cells are essential for the development of GCs, raising the question which of these populations was responsible for provision of T_{FH} cell function. In order to investigate this, $TCR^{bd-/-}$ recipients were reconstituted with SAP^{-ve} CD4 T cells from $SAP^{-/-}$ transgenic mice. SAP signaling is essential for the development of the CD4 T_{FH} cell subset (Crotty et al., 2003; Kamperschroer et al., 2006). Thus if T_{FH} cell function is provided by the recipient CD4 T cell population, GC activity would not be observed upon transfer of bm12 CD4 T cells into a $TCR^{bd-/-}$ BL6 recipient reconstituted with SAP^{-ve} BL6 CD4 T cells.

On reconstitution of T cell deficient recipient with bm12 ($2x10^6$) and SAP^{-ve} BL6 ($4x10^6$) CD4 T cells simultaneously, splenic GC reactions were not detectable. Interestingly, however, high strong autoantibody responses were generated initially after transfer, but declined rapidly from week 3 onwards (figure 4.8), in keeping with a self-limiting extrafollicular response. The early autoantibody response in this group was greater than that observed in T cell deficient recipients challenged with purified bm12 CD4 T cells (figure 4.5), possibly suggesting that the recipient $SAP^{-/-}$ CD4 T cells are contributing to the development of the extrafollicular response.

a.



b.

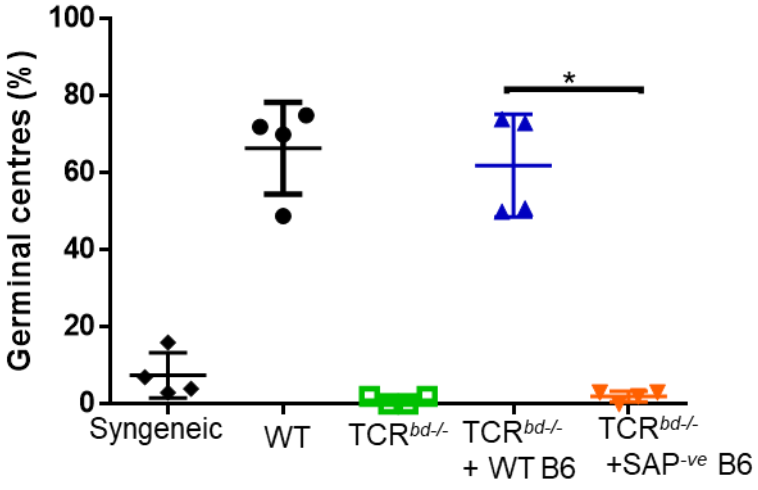


Figure 4.8: Failure of the B cell follicles to differentiate into GCs on restoration of T cell deficient recipients with bm12 and SAP^{-ve}.BL6 CD4 T cells with short lasting autoantibodies.

a. Relative anti-nuclear autoantibody levels as measured by Hep-2 indirect immunofluorescence in TCR$^{bd-/-}$ recipients reconstituted with donor and SAP$^{-/-}$ BL6 CD4 T cells demonstrating short lasting autoantibodies which was initially very high and then it starts declining after week 5. Autoantibodies were statistically significantly higher in T cell deficient recipients reconstituted with SAP^{-ve} CD4 T cells compared to WT response.

b. Quantification of GCs in splenic sections of the recipients 7 weeks after adoptive transfer of bm12 CD4 T cells into TCR$^{bd-/-}$ reconstituted with SAP^{ve-} BL6 CD4 T cells demonstrating that GCs were significantly less than WT responses.

*P < 0.05 and **P < 0.01, Two way ANOVA for a and Mann Whitney for b was used.

Hence, donor CD4 T cells are important for initiating recipient humoral autoimmunity, but thereafter, recipient CD4 T cells provide help for the development and maintenance of long-lasting GC reactions. Then I sought to investigate which of the population of CD4 T cells localises to GCs.

Because bm12 is a natural mutant of BL6 WT animal and all of the cells are CD45.2 positive in both of them, CD45.1 BL6 transgenic mice were used as a recipient in order to differentiate between donor and recipient CD4 T cells.

Column purified bm12 (CD45.2) CD4 T cells were adoptively transferred into CD45.1.BL6 recipients as per methods (section 2.2.1.5). Recipient splenic sections were examined for the presence of GCs and CD4 T cells at various time points (figure 4.9). Confocal staining was carried out in order to examine the anatomical localisation of donor and or recipient CD4 T cells within GCs. Firstly presence of CD4 T cells within the GCs was confirmed by staining recipients' splenic sections with anti-CD4, anti-B220 and anti-GL7 antibodies and secondly donor CD4 T cells were identified within GCs by staining recipients' splenic sections with anti-CD45.2 (donor), anti-CD4 and anti GL7 antibodies. Because all the recipient cells were CD45.1 positive including B cells, T cells and DCs, it was not possible to see the recipient CD45.1 CD4 T cells in GCs. Hence, recipient splenic sections were stained with anti-GL7, anti-CD4 and anti-CD45.2 antibodies, and the fraction of CD4 T cells which was not positive for CD45.2, was considered as recipient (CD45.1) in origin.
Of note, I was unable to combine more than three fluorochromes on one tissue section during staining due to availability of only three fluorochrome filters on our confocal microscope.

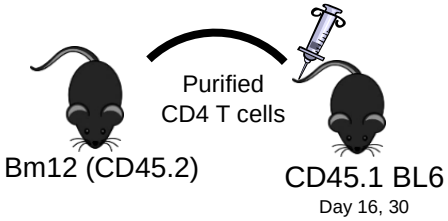


Figure 4.9. Experimental model showing adoptive transfer of donor bm12 (CD45.2) CD4 T cells into CD45.1 BL6 recipients

Splenic sections of recipients were stained with anti-CD4, anti-B220 and anti-GL7 antibodies to identify CD4 T cells in GCs or with anti-CD45.1/anti-CD45.2, anti-CD4 and anti-GL7 antibodies to see the presence of donor or recipient CD4 T in the GCs.

Adoptive transfer of purified donor CD4 T cell ($2x10^6$) was carried out into CD45.1 BL6 recipient. Recipient spleens were harvested at day 16 (week 2), (the time when GCs were first observed in BL6 WT recipients (figure 3.7)), and splenic sections examined for the presence of donor (CD45.2) and recipient (CD45.1) CD4 T cells within the GCs. On staining, CD4 T cells were identified in periarteriolar lymphoid sheaths **(**PALS), B cells follicles and GC (figure 4.10 i). Staining with anti-CD45.2 (donor) and anti-CD45.1 (recipient) antibodies revealed the presence of both donor and recipient CD4 T cells within the GC areas (figure 4.10 ii). Detailed analysis of stained sections demonstrated that most of the CD4 T cells localising to GC areas were of donor origin. There was, however, also a population of CD45.2 negative CD4 T cells within the GC. Furthermore, the presence of recipient CD45.1 positive CD4 T cells was seen in PALS area, B cell follicles and in GCs areas on staining splenic sections with anti-CD45.1, anti-CD4 and anti-GL7 antibody. Of note, as all the recipient cells carry CD45.1 marker on their surface, CD45.1 staining demonstrated a good architecture of follicles and PALS area as shown in right hand panel of figure 4.10 ii.

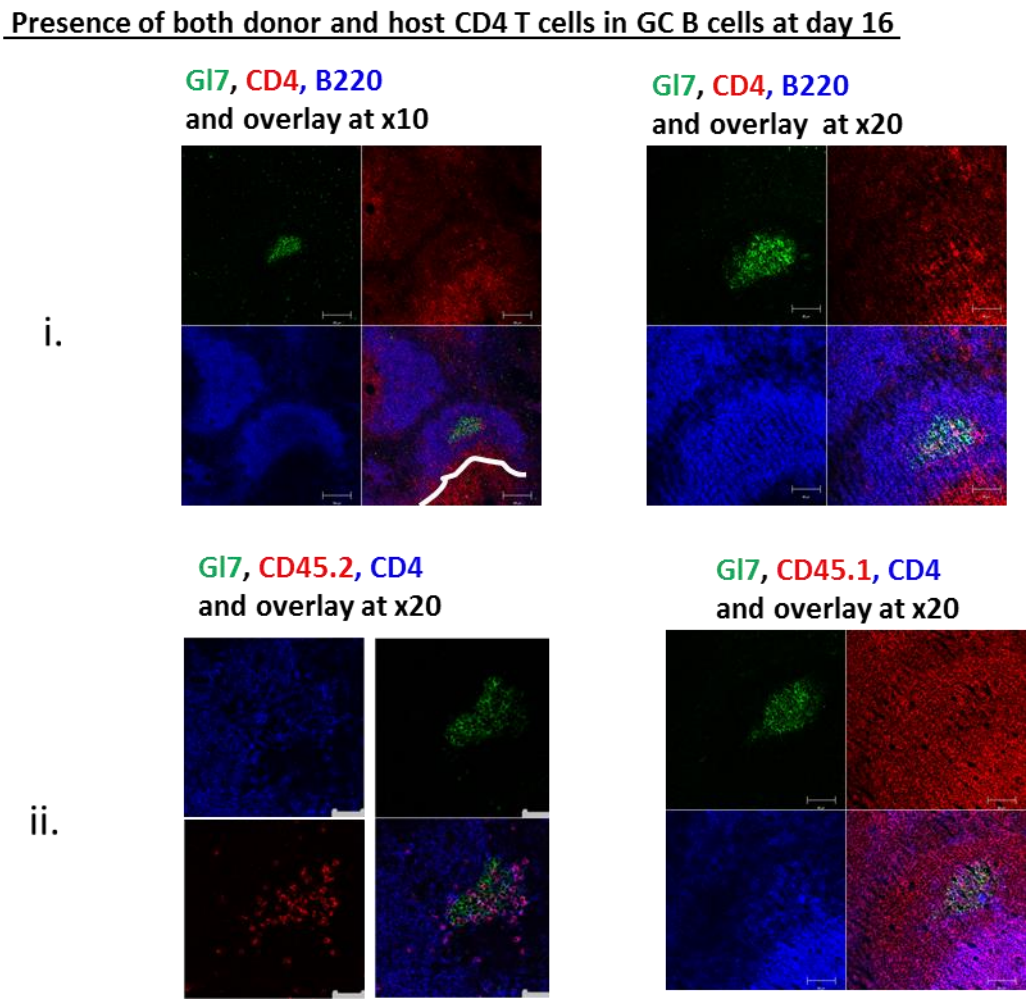


Figure 4.10. Confocal immunofluorescence to identify donor and recipient CD4 T cells in GC B cells after adoptive transfer of bm12 CD4 T cells into CD45.1 recipients at day 16 confirming the presence of both donor and recipient CD4 T cells in GC B cell area. (Figure is representative of 4 animals). GL7 is green, CD4/CD45.2/CD45.1 is red and B220 is blue coloured staining. i). Presence of CD4 T cells (red) in PALS area (right lower end corner-in red, highlighted with white line) and in GC B cells (green) as identified by staining with anti CD4 antibody (red), anti GL7 antibody (green) and anti B220 antibody (blue).

ii). Presence of donor (CD45.2) CD4 T cells (red) in GC B cells (green) as identified by staining with anti-CD45.2 antibody (red), anti-GL7 antibody (green) and anti-CD4 antibody (blue) in the left sided figure (ii).However, on the right side, presence of recipient CD4 T cells is shown as stained with anti-CD45.1 antibody (red), anti-CD4 antibody (blue) and anti-GL7 antibody (green). Scale bar is 100µm for figure i) left handed panel and 50µm for the rest of the figures.

At day 30 (week 4) after adoptive transfer of bm12 CD4 T cells into CD45.1 recipients, fewer donor cells were seen in GC areas as demonstrated by fewer of the CD4 T cells were found to be positive for CD45.2 as shown below in figure 4.11 ii.

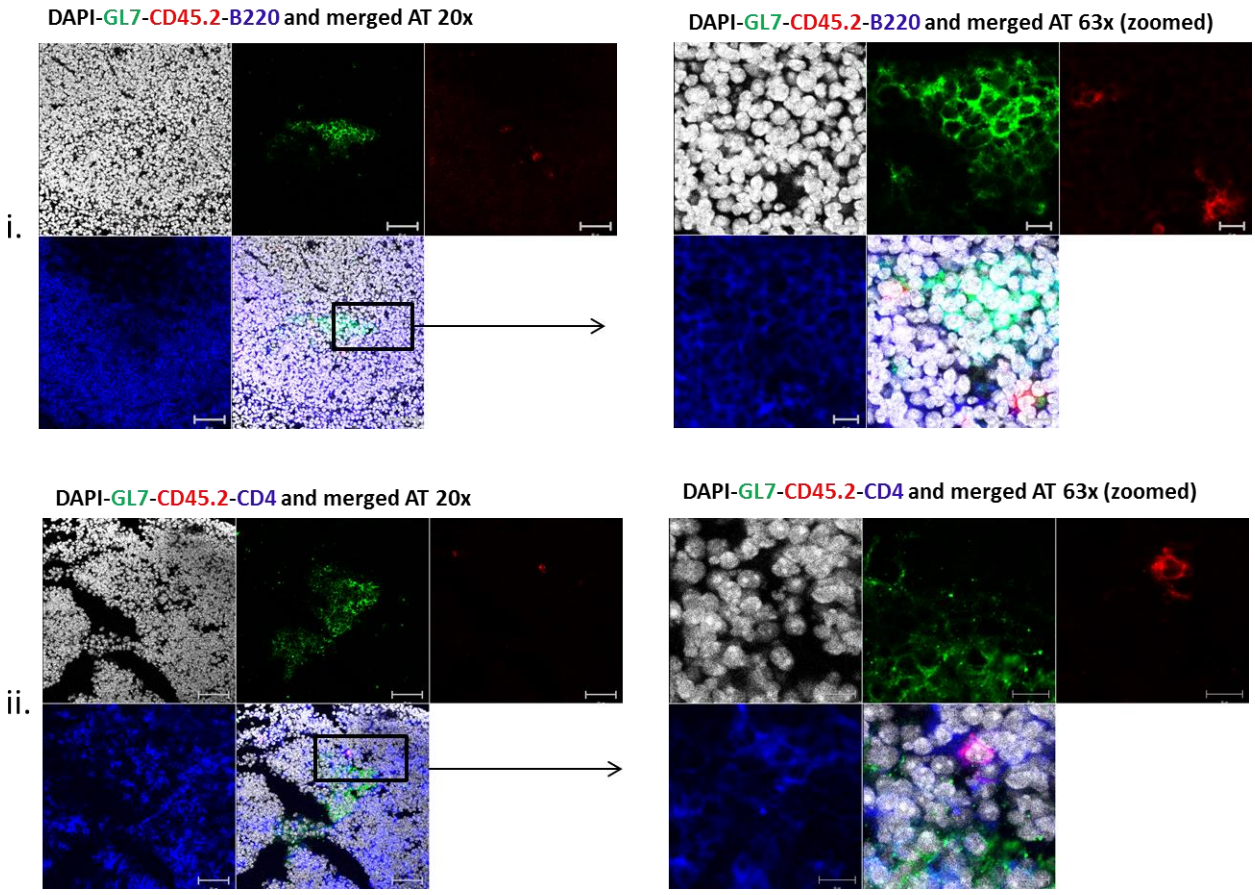
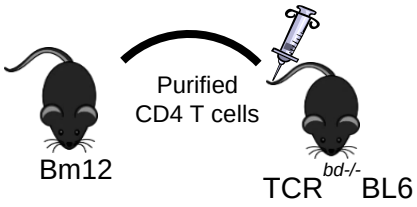


Figure 4.11. Confocal immunofluorescence to identify donor and recipient CD4 T cells in GC B cells after adoptive transfer of bm12 CD4 T cells into CD45.1 recipients at day 30 confirming the presence of both donor and recipient CD4 T cells in GC B cell area, fewer donor CD45.2 CD4 T cells than recipient non CD45.2 positive CD4 T cells in GC B cells. DAPI is black, GL7 is green, CD45.2 is red and B220 is blue coloured staining. i). Presence of CD45.2 positive cells (red) in GC B cells (green) as identified by staining with anti-CD45.2 antibody (red), anti-GL7 antibody (green) and anti-B220 antibody (blue). Germinal centre (green) area in the left panel is zoomed in to identify the presence of donor CD45.2 (red) cells in GC B cells area to 5 times as shown in the right panel.

ii). Presence of donor (CD45.2) CD4 T cells (red) in GC B cells (green) as identified by staining with anti-CD45.2 antibody (red), anti-GL7 antibody (green) and anti-CD4 antibody (blue) in the left sided figure. Germinal centre (green) area in the left panel is zoomed to identify the presence of donor CD45.2 positive (red) CD4 T cells (blue) in GC B cells area to 5 times as shown in the right panel.

Scale bar is 50µm for left handed panel and 10µm for right handed panel figures.

As shown above in figure 4.5 that B cells in T cell deficient mice did not differentiate into GCs after being challenged with bm12 CD4 T cells, this raised a question of where these donor bm12 CD4 T cells were homing in these recipients. To investigate this, $TCR^{bd-/-}$ recipient splenic sections were stained with anti-CD4 and anti-B220 antibody and looked for their localisation on confocal at 7 weeks post adoptive transfer of cells. On staining, CD4 T cells which are essentially of donor origin, localised in PALS area and margins of B cell follicles as shown below in figure 4.13.



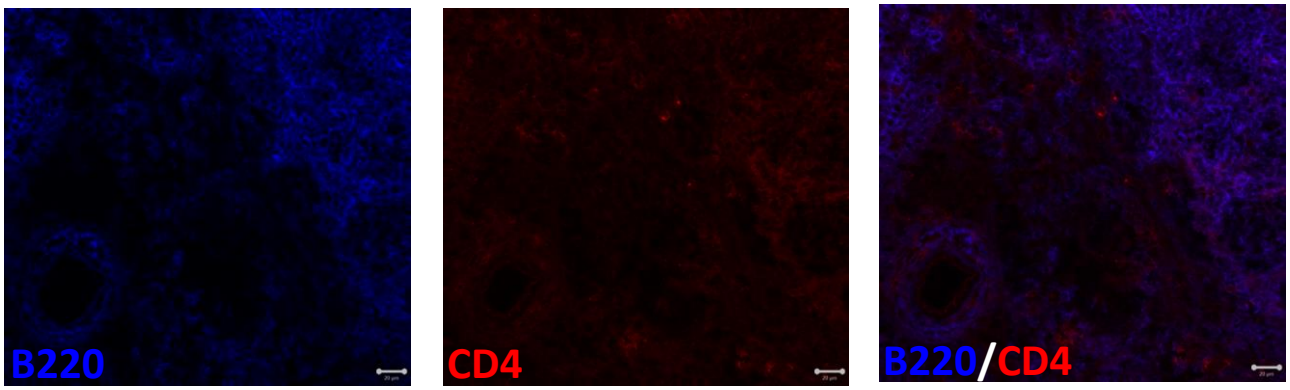


Figure 4.13. Confocal immunofluorescence of recipient TCR$^{bd-/-}$ recipient splenic to identify CD4 T cells in B cell follicles at week 7 post adoptive transfer of bm12 CD4 T cells, demonstrating presence of CD4 T cells in PALS area and primary B cells follicles demonstrated by staining with anti-CD4 and anti-B220 antibody. Scale bar is 20µm.

This set of experiments demonstrate that at an early time point, both donor and recipient CD4 T cells were present in GC areas, suggesting that either of them or both populations would be acting as a T_{FH} cells. Localisation of donor and recipient CD4 T cells at a late time point was carried out in transplantation experiments which will be discussed in the next chapter (section 5.4.2. and figure 5.4b).

4.3.3. Blocking SAP signalling in donor and recipient CD4 T cells

To definitively find out that whether donor or recipient CD4 T cells are functioning as T_{FH} cells in maintaining GC autoimmunity in this model, adoptive transfer of column purified bm12 ($2x10^6$) or SAP^{-ve}.bm12 CD4 T cells ($2x10^6$) into SAP^{-ve} BL6 or WT BL6 recipients was carried out respectively. Autoreactive humoral responses were assessed by hep-2 indirect immunofluorescence assay of circulating autoantibody, and by quantitation of splenic GC activity.

4.3.3.1. Blocking SAP signalling on recipient CD4 T cells abrogates long term GCs

Adoptive transfer of bm12 CD4 T into SAP^{-ve} B6 (SAP^{-ve}.B6) recipients provoked a short lasting autoantibody response which was significantly low compared to WT response (figure 4.14a). However, recipient splenic sections showed the development of germinal centres at early time point (week 3) but at late time point (week 7) GC response was not above the base line (figure 4.14b).

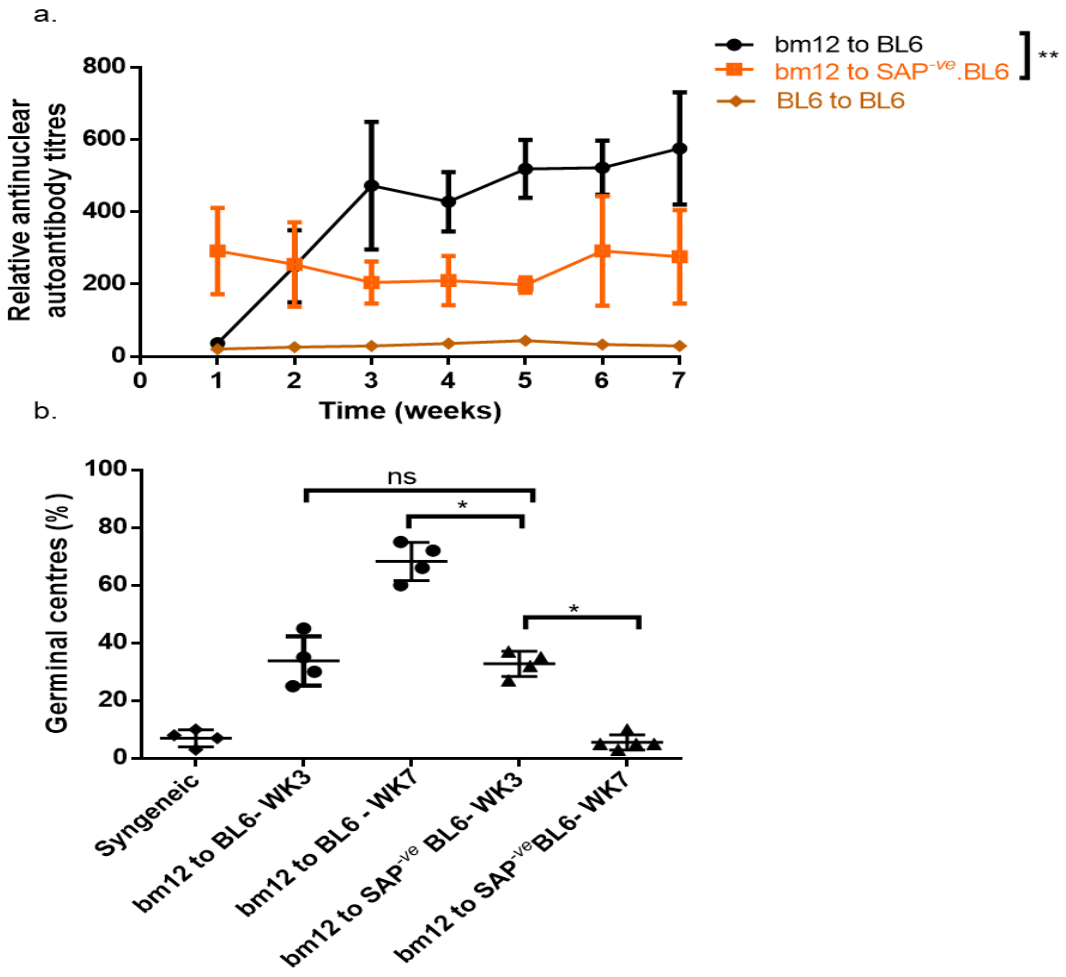


Figure 4.14: Failure of B cell follicles to sustain long term germinal centres after adoptive transfer of bm12 CD4 T cells into SAP^{-ve}.BL6 (SAP^{-ve}.BL6) recipients

a. Relative anti-nuclear autoantibody levels as measured by Hep-2 indirect immunofluorescence in SAP^{-ve}.BL6 recipients following adoptive transfer of bm12 CD4 T cells demonstrating significantly low levels of autoantibodies compared to WT response (two way ANOVA, p= 0.008).

b. Quantification of GCs in splenic sections of the SAP^{-ve}.BL6 recipients at early (week 3) and late time points (week 7) after adoptive transfer of bm12 CD4 T cells, demonstrating that GCs were less than WT response but did not reach statistical significance at week 3 (p=0.142), but at week 7 this difference achieved significance due to amelioration of GCs (Mann Whitney test, p= 0.015).

Data represent mean and SD of n = 4 mice per group.

This confirmed that inhibition of SAP signalling in the recipients abrogated long term GC autoantibody response. Furthermore, it highlighted that donor CD4 T cells may be providing a limited and transient T_{FH} function for GC autoimmunity.

4.3.3.2. Blocking SAP signalling on donor CD4 T cells failed to inhibit long term GCs

Having established that inhibition of SAP signalling in the recipient CD4 T cells resulted in abrogation of long term GCs in the recipients, I next sought to investigate the role of inhibition of SAP signalling in donor CD4 T cells. This was carried out by using donors in which SAP signalling was knocked out by crossing bm12 males with $SAP^{-/+}$ females as discussed in methods section 2.1.1 and 2.2.10. After the confirmation of SAP^{-ve}.bm12 status of donors on gel electrophoresis as described in methods section 2.2,1 and 2.2.10, they were used for experiments.

Hep-2 indirect immunofluorescence of recipient sera from SAP^{-ve}.bm12 groups demonstrated long lasting autoantibody which was in consistent with the presence of GCs in recipient splenic sections at 7 weeks post adoptive transfer. This humoral autoimmune response was comparable with the WT response (figure 4.15).

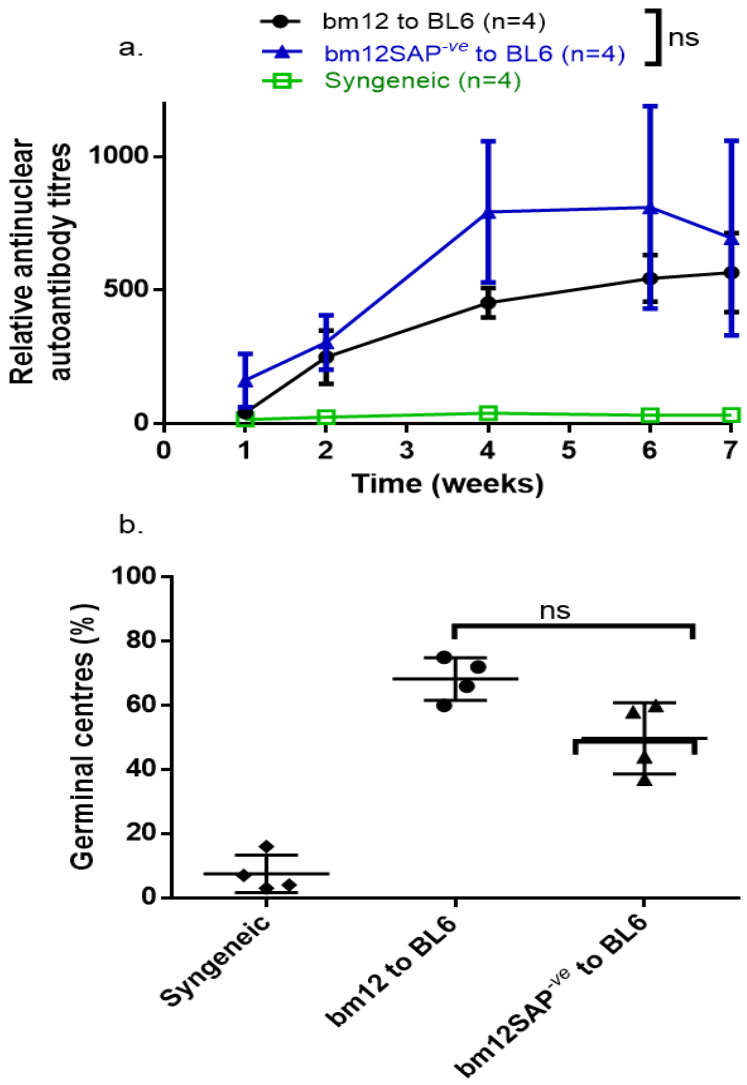


Figure 4.15. Development of autoantibodies and long term GCs in BL6 recipients following adoptive transfer of SAP^{-ve}.bm12 purified CD4 cells (2x10^6).

a. Relative anti-nuclear autoantibody levels as measured by Hep-2 indirect immunofluorescence in BL6 recipients following adoptive transfer of donor SAP^{-ve}.bm12 CD4 T cells demonstrating comparable autoantibodies titres compared to WT response (two way ANOVA, p= 0.148).

b. Quantification of GCs in splenic sections of the BL6 recipients at week 7 after adoptive transfer of SAP^{-ve}.bm12 CD4 T cells demonstrating that GCs were comparable with that of WT (Mann Whitney test, p= 0.190).

Data represent mean and SD of n = 4 mice per group.

To further examine this critical role of the recipient CD4 T cell population in maintaining humoral GC autoimmunity, WT BL6 mice were challenged intravenously with $2x10^6$ column purified CD4 T cells from 'bm12.K^d' mice (that express additional transgenic H-$2K^d$ alloantigen). Four weeks later, CD4 T cells were purified from these challenged mice and transferred into secondary naïve BL6 recipients. We have recently reported that, as a consequence of the mismatched I-A^{bm12} antigen, purified bm12.K^d CD4 T cells generate graft versus host responses in BL6 recipients, resulting in long-lived anti-K^d-alloantibody and autoantibody production, despite the transferred bm12.K^d CD4 T cells being killed within the first week after transfer by host adaptive immune recognition of the H-$2K^d$ alloantigen (Harper et al., 2016). Thus, BL6 mice were culled 4 weeks after challenge with bm12.K^d CD4 T cells (by which time only recipient-strain CD4 T cells will have survived) and column purified CD4 T cells from these mice was adoptively transferred into secondary naive BL6 recipients. Serum was sampled weekly from the secondary recipients, and their spleens assessed at 7 weeks for GC activity. As shown in Figure 4.16, adoptive transfer of purified CD4 T cells from bm12.K^d-challenged mice provoked GC humoral autoimmune responses in the secondary recipients (Figure 4.16). This confirms that recipient CD4 T cells are not only maintaining the GC response, but can trigger humoral autoimmunity upon transfer into naïve mice.

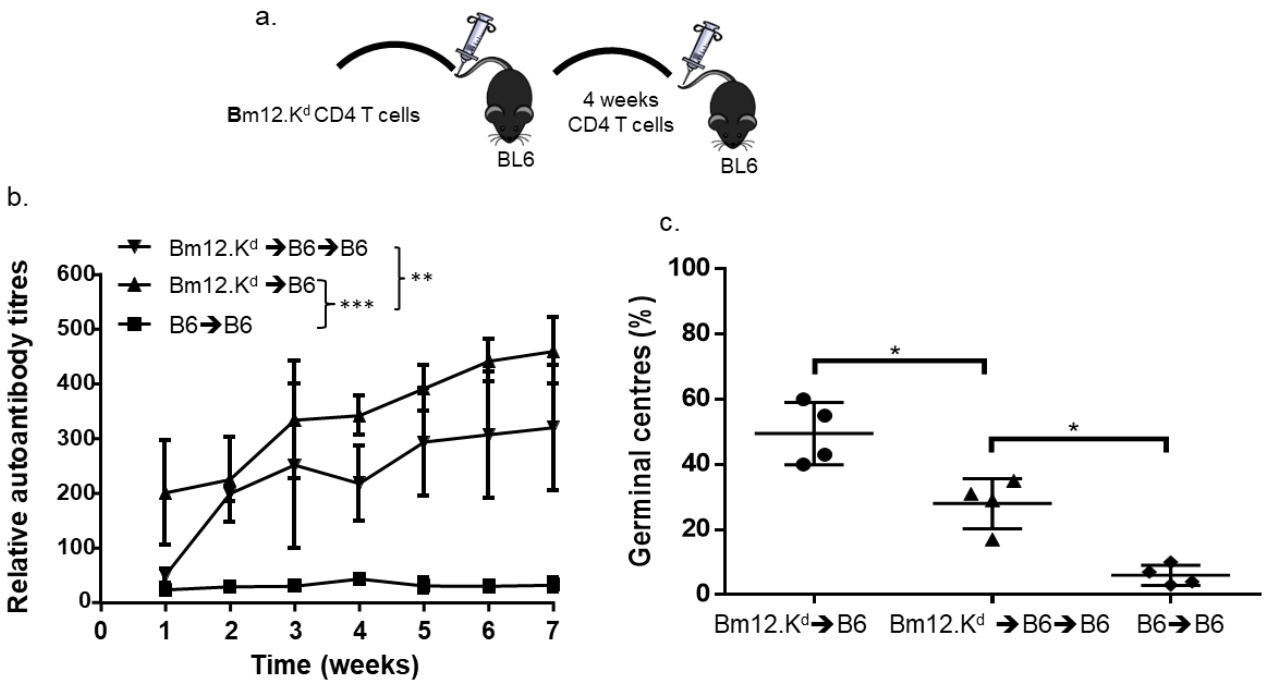

a.
Bm12.K^d CD4 T cells
BL6
4 weeks
CD4 T cells
BL6
b.
Bm12.K^d →B6→B6
Bm12.K^d →B6
B6→B6
**
***
Relative autoantibody titres
Time (weeks)
c.
Germinal centres (%)
*
*
Bm12.K^d→B6
Bm12.K^d →B6→B6
B6→B6

Figure 4.16. BL6 WT CD4 T cells potential for initiating and maintaining autoimmunity in secondary naïve WT BL6 host.

a. Column purified bm12.K^d CD4 T cells ($2x10^6$) were transferred into naïve BL6 recipients, and four weeks later, CD4 T cells were purified from these challenged mice and ($2x10^6$ CD4 T cells) transferred into secondary naïve BL6 recipients (Bm12.K^d ➔BL6➔BL6).

b. Hep-2 indirect immunofluorescence demonstrating the development of long lasting autoantibodies in BL6 recipients of BL6 CD4 T cells from BL6 recipients of bm12.Kd CD4 T cells (Bm12.K^d ➔BL6➔BL6) which is significantly higher than syngeneic BL6 CD4 T cells (BL6➔BL6) but less than Bm12.K^d➔BL6 responses (p=.003).

c. Quantification of GCs in splenic sections of the BL6 recipients of BL6 CD4 T cells from BL6 recipients of bm12.K^d CD4 T cells (Bm12.K^d ➔BL6➔BL6) at week 7 on immunofluorescence staining by staining with anti-GL7 for germinal centres and anti-B220 for B cell follicles. On quantification, it showed that GCs were significantly higher than syngeneic group but less than Bm12.K^d➔BL6.

Data represent mean and SD of n = 4 mice per group, **P* < 0.05, ***P* < 0.01 and ****P* < 0.001; 2-way ANOVA in ii and Mann-Whitney test in iii**.**

4.3.4. Germinal centres are central to diversification of humoral responses

Having established that donor CD4 T cells initiated recipient autoantibody responses that were then maintained as a GC reaction by T_{FH} cells of recipient origin, I sought to address the following:

1. Do the autoreactive humoral responses in BL6 recipients of bm12 CD4 T cells diversify with time, as occurs in BL6 recipients of bm12 heart grafts?
2. If so, is this epitope diversification mediated by a germinal centre response?

In order to address the first question, anti-vimentin autoantibody was measured and compared by anti-vimentin antibody ELISA at an early and late time point in BL6 recipients of bm12 CD4 T cells as described in method section (2.2.5.3). On analysis, it was found that there was a surge in anti-vimentin autoantibody at a late time point compared to early time point (figure 4.17). These findings were in accordance which were seen by bm12 allografts into BL6 as described in the last chapter in section 3.3.6.2.

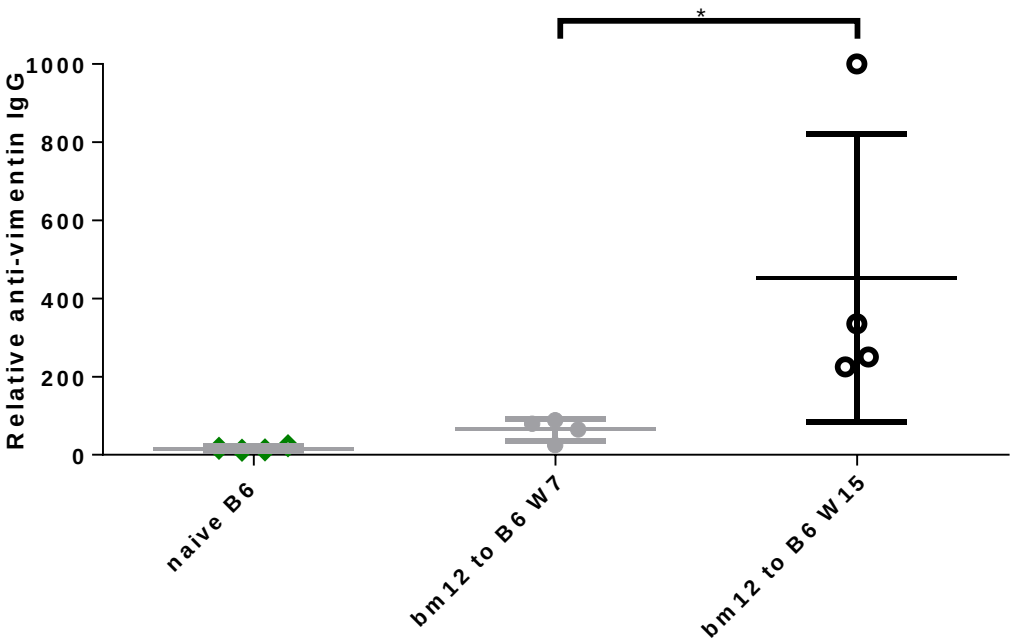


Figure 4.17. Anti-vimentin autoantibody responses as measured by ELISA at early and late time point

Anti-vimentin ELISA demonstrating that relative levels of anti-vimentin IgG antibody were significantly higher at week 15 compared to week 7 following transfer of purified bm12 CD4 T cells into naïve BL6 recipients.

Data represent mean and SD of n = 4 mice, (p= Mann-Whitney test).

To address the second question, sera from the challenged groups were tested for the presence of vimentin autoantibody.

On analysis, anti-vimentin autoantibody levels were not above base line in $TCR^{bd-/-}$ mice challenged with bm12 CD4 T cells, nor in challenged SAP^{-ve} BL6 mice. In contrast, late anti-vimentin (week 15) responses were detected in challenged $TCR^{bd-/-}$ mice that had also been reconstituted with naïve BL6 CD4 T cells – the only group in which long-lasting GC responses were observed (figure 4.18).

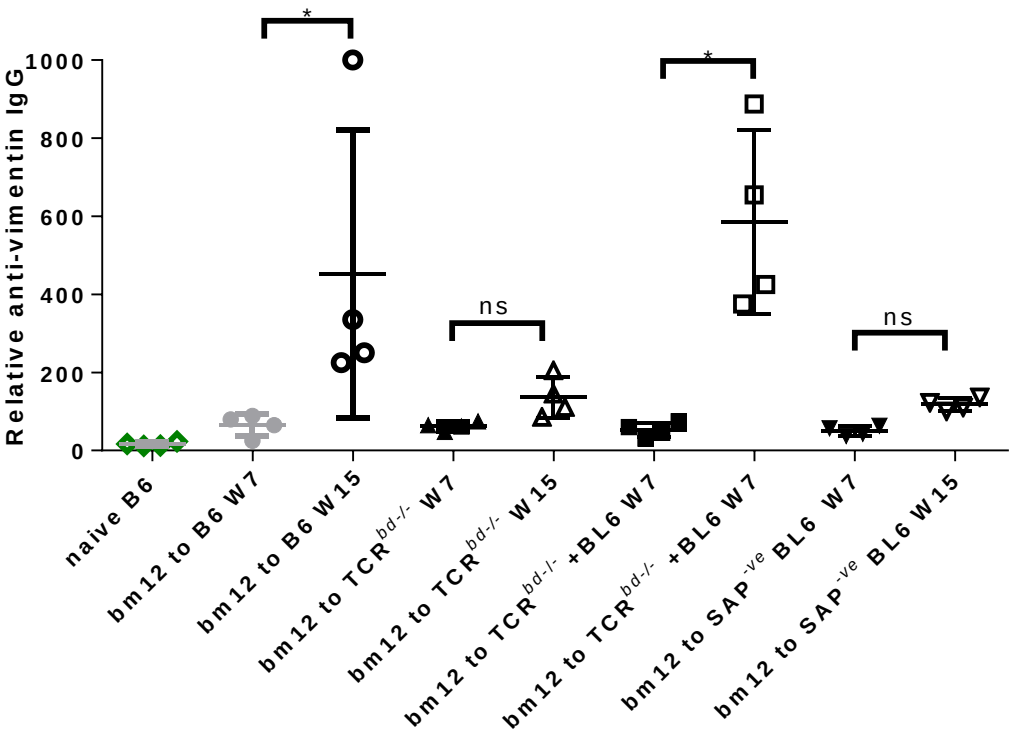


Figure 4.18. Diversification of humoral responses as demonstrated by development of anti-vimentin autoantibody at early and late time points after adoptive transfer of bm12 CD4 T cells into BL6 / TCR$^{bd-/-}$ / TCR$^{bd-/-}$+ BL6 CD4 T cells / SAP^{-ve} BL6 recipients

Anti-vimentin ELISA demonstrating the relative levels of anti-vimentin IgG antibody at early (week 7) and late time points (week 15). It showed that significantly higher anti-vimentin levels in BL6 (WT) and TCR$^{bd-/-}$ +BL6 recipients at week 15 compared to week 7 but not in TCR$^{bd-/-}$ and SAP$^{-/-}$ BL6 recipients.

Data represent mean and SD of n = 4 mice per group, n= not significant,*$P < 0.05$, (Mann-Whitney test).

This experiment demonstrated that GCs may be playing a major role in diversification of humoral response.

4.3.5. Monoclonal population of CD4 T cells is capable of driving diversification of humoral response in donor induced autoimmunity

The findings raise the interesting question of precise antigen-specificity of the recipient CD4 T cells that provide secondary T_{FH} cell help in our model.

Essentially, host T_{FH} cells are recognising the processed autoantigens presented in the context of MHC class II by the activated autoreactive B cells and result in the development of autoreactive GCs. These autoreactive GCs then lead to inter-molecular epitope diversification, to encompass, along with other targets, responses against vimentin autoantigen. However, if we replace WT host CD4 T cells with TCR75 CD4 T cells (that recognise the dominant peptide epitope of the H2.K^d MHC class I antigen in the context of I-A^b) then K^d specific B cells would present the K^d antigen to TCR75 CD4 T cells and result in the development of only Kd specific GCs and there should be no anti-vimentin autoantibody response.

In order to investigate this, $TCR^{bd-/-}$ or WT B6 mice were reconstituted with TCR75 T cells/ SAP^{-ve}. TCR75 CD4 T cells and simultaneously adoptively transferred column purified CD4 T cells from bm12 donor mice that express additional H-2K^d class I alloantigen as a transgene (bm12.K^d). The experimental model is shown below in figure 4.19.

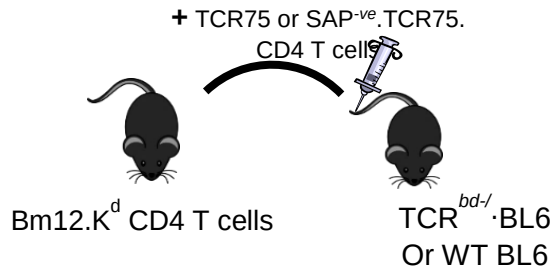


Figure 4.19. Experimental model in which TCR$^{bd-/-}$ or WT BL6 recipients were reconstituted with purified TCR75 or TCR75 SAP$^{-/-}$ CD4 T cells (1x10^3 TCR75 CD4 T cells) and simultaneously with purified bm12.Kd CD4 T cells (2x10^6).

Sera from BL6 recipients of bm12.K^d CD4 T cells demonstrated development of anti-vimentin autoantibody response at a late time point (figure 4.20) similar to that observed in bm12 to BL6 cell adoptive transfer experiment. In this groups, I have already shown that recipients developed LL autoantibody and alloantibody, which were consistent with the development of GCs (figure 4.16 a, b and c).

These results demonstrate that Kd specific and autoantigen specific T_{FH} cells were helping the respective populations of activated B cells to produce K^d alloantibody and autoantibodies respectively.

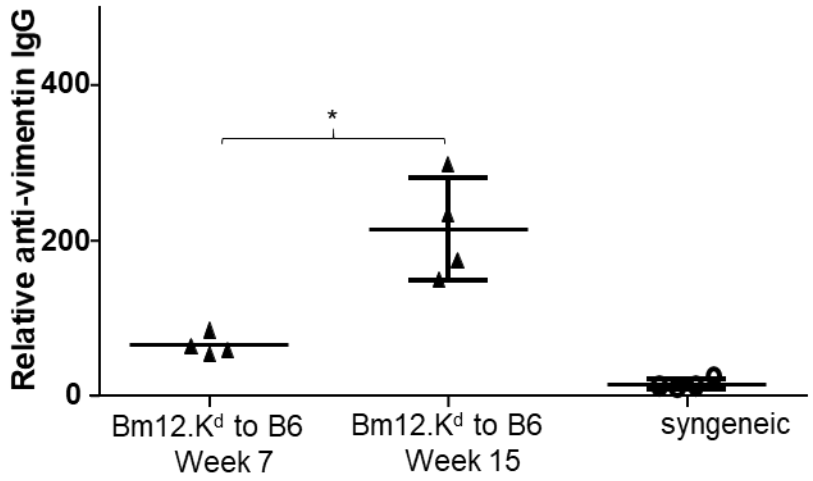


Figure 4.20. Development of late anti-vimentin autoantibody response in BL6 recipients of bm12.K^{d} CD4 T cells.

Anti-vimentin ELISA demonstrating development of significantly high levels of anti-vimentin autoantibody at week 15 compared to week 7 (p=.02)

Data represent mean and SD of n = 4 mice per group,*$P < 0.05$, Mann-Whitney test.

After having established that BL6 recipients of bm12.K^d CD4 T cells reproduced diversification of autoantibody in the same manner as that observed in bm12 to BL6 model, the next step was to adoptively transfer bm12.K^d CD4 T cells into $TCR^{bd-/-}$ recipients and to examine humoral response in the recipients and diversification.

On adoptive transfer of bm12.K^d CD4 T cells into $TCR^{bd-/-}$, recipient sera showed development of autoantibody and alloantibody which was in consistent with our previous work (Harper et al., 2016). But B cells did not differentiate into GCs as demonstrated by GL7 negative B cell follicles. Despite the presence of autoantibody, anti-vimentin autoantibody was not above the base line (figure 4.21). These findings were in consistent with bm12 to $TCR^{bd-/-}$ experiment results that in the absence of secondary host T_{FH} cells, recipients did not develop GCs and there was no late anti-vimentin autoantibody response (figure 4.18).

The next step was to investigate that whether monoclonal population of CD4 T cells is sufficient enough to drive this diversification. In order to investigate this, $TCR^{bd-/-}$ were reconstituted with CD4 T cells from TCR75 as shown above in figure 4.19. On reconstitution of these $TCR^{bd-/-}$ recipient with TCR75 CD4 T cells, GCs were restored with LL autoantibody and anti-Kd antibody in the recipients. Interestingly, the anti-vimentin autoantibody formed earlier compared to WT scenario (bm12.Kd to BL6), demonstrating stronger help provided by monoclonal population of CD4 T cells for the development of anti-vimentin autoantibody compared to WT (figure 4.21).

Furthermore, this ability of monoclonal population of CD4 T cells to drive humoral response diversification in GVH, was confirmed by reconstituting $TCR^{bd-/-}$ recipients with SAP^{-ve} TCR75.CD4 T cells and challenged with bm12^{Kd} cell simultaneously (figure 4.19). These SAP^{-ve} TCR75.CD4 T cells were monoclonal population of CD4 that recognise the dominant peptide epitope of the H2.Kd MHC class I antigen in the context of I-A^b but were not able to provide T_{FH} cell function for the development of GCs due to inhibition of SAP signalling. Following adoptive transfer, recipient sera showed the presence of autoantibody and anti-K^d antibody but B cells did not differentiate into GCs and there were no anti-vimentin autoantibody (figure 4.21).

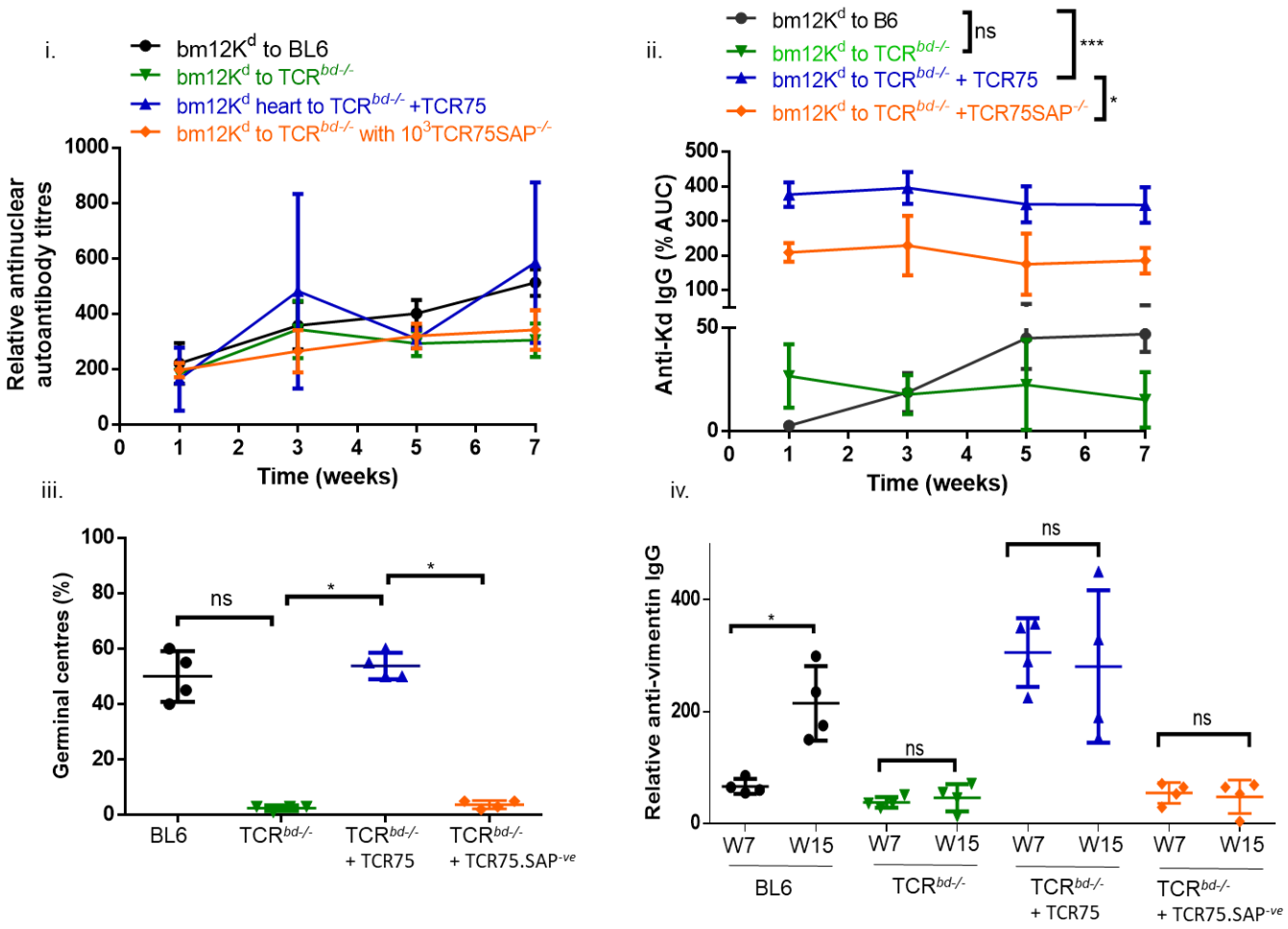

i.
bm12K^d to BL6
bm12K^d to TCR^bd-/-
bm12K^d heart to TCR^bd-/- +TCR75
bm12K^d to TCR^bd-/- with 10^3TCR75SAP^-/-
Relative antinuclear autoantibody titres
Time (weeks)
ii.
bm12K^d to B6
bm12K^d to TCR^bd-/-
bm12K^d to TCR^bd-/- + TCR75
bm12K^d to TCR^bd-/- +TCR75SAP^-/-
ns
***
*
Anti-Kd IgG (% AUC)
Time (weeks)
iii.
Germinal centres (%)
ns
*
*
BL6
TCR^bd-/-
TCR^bd-/- + TCR75
TCR^bd-/- + TCR75.SAP^-ve
iv.
Relative anti-vimentin IgG
*
ns
ns
ns
W7
W15
BL6
TCR^bd-/-
TCR^bd-/- + TCR75
TCR^bd-/- + TCR75.SAP^-ve

Figure 4.21. Restoration of autoantibody response, Kd antibody and GCs on reconstitution of TCR$^{bd-/-}$ with bm12.K^{d} CD4 T cells and TCR75 CD4 T cells but not with SAP^{-ve} TCR75.CD4 T cells

i. Relative anti-nuclear autoantibody levels as measured by Hep-2 indirect immunofluorescence in TCR$^{bd-/-}$ recipients of bm12.K^{d} CD4 T cells with or without TCR75 CD4 T cells or SAP^{-ve} TCR75.CD4 T cells, demonstrating development of long lasting autoantibodies in all the groups which are comparable in all the groups.

ii. Kd ELISA demonstrating development of relative anti-Kd antibodies in TCR$^{bd-/-}$ recipients reconstituted with SAP$^{-/-}$ TCR75.CD4 T cells demonstrating development LL anti-Kd antibody which was significantly less than TCR$^{bd-/-}$ recipients reconstituted with TCR75 but more than WT and TCR$^{bd-/-}$ recipients.

iii. On quantification of GCs, germinal centres were significantly lower in TCR$^{bd-/-}$ recipients reconstituted with SAP^{-ve} TCR75.CD4 T cells compared to WT and TCR$^{bd-/-}$ recipients reconstituted with TCR75 CD4 T cells.

iv. Anti-vimentin ELISA demonstrating that anti-vimentin autoantibody was not above the base line in TCR$^{bd-/-}$ recipients reconstituted with SAP^{-ve} TCR75.CD4 T cells while in TCR$^{bd-/-}$ reconstituted with TCR75 CD4 T cells demonstrated high levels of anti-vimentin even at week 7.

Data represent mean and SD of n = 4 mice per group, ns=not significant, *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, Two way ANOVA for figure i, ii and Mann-Whitney test for iii, iv**.**

This experimental work revealed that monoclonal population of CD4 T cells was sufficient to drive diversification of autoimmune humoral response in recipients once triggered by donor CD4 T cells. The possible explanation may be that initial transient autoreactive GC triggered by bm12K^{d} cells may be sustained by TCR75 CD4 T cells.

4.3.6. Factors which may be preventing donor bm12 CD4 T cells to acquire T_{FH} function

Because donor bm12 CD4 T cells do not undergo T_{FH} cell differentiation (figures 4.14 and 4.15), this raised the intriguing question as to why not; bm12 mice readily mount conventional T cell-dependent antibody responses (Huygen et al., 1993; McIntyre and Seidman, 1984). We think there are two likely explanations.

1. Firstly, for any particular donor alloreactive bm12 CD4 T cell clone, its target epitope is likely to be expressed at much higher frequency on the surface of the host (BL6) B cells and DCs, than would normally be the case for conventional recognition of a self-restricted peptide epitope. Because in GVH, donor CD4 T cells interact with all the MHC class II complexes in the recipient irrespective of the bound peptide, hence the donor precursor frequency that responds to a particular MHC class II alloantigen is 100 to 1,000 fold greater than for the response against conventional, self-restricted peptide (Ali et al., 2016; Macdonald et al., 2009). During this process, donor CD4 T cells may have non-productive interactions with non-B cell APCs, most probably with dendritic cells (DCs). These non-productive interactions may result in their inability to function as a T^{FH} cell. Contrary to this hypothesis, Deenick et al has suggested that prolonged DC interaction can drive T_{FH} differentiation (Deenick et al., 2010).
   In this respect there is one potential possibility that successive interaction of donor CD4 T cells with dendritic cells (DCs) may result in progressive reduction in Bcl-6 expressed by donor CD4 T cells. Hence donor CD4 T cells do not acquire T_{FH} cell signature markers which prevent their entry and retention into B cell follicles for development of long term germinal centres. Hence, we hypothesised that overexpression of Bcl-6 on donor bm12 CD4 T cells will make them to function as T_{FH} cells for development of GC response.
2. Secondly, although a remarkably diverse set of bm12 CD4 T cell clones are activated by GVH recognition of recipient MHC class II, no individual clone binds with sufficiently-strong affinity to enable differentiation into follicular helper CD4 T cells. It has been suggested that T cell receptor antigen binding strength determines the fate of CD4 T cells to acquire a durable T_{FH} cell function (Fazilleau et al., 2009). In order to investigate this concept of antigen binding strength in direct allorecognition, we used TCR75 CD4 T cells' ability to recognise I-A^b-restricted K^d peptide epitope in a

direct allorecognition fashion.TCR75 CD4 T cells are a monoclonal population of CD4 T cells and we have shown recently that they acquire T_{FH} cell function when recognising dominant K^d- peptide in the context of I-A^b in an indirect fashion (Conlon et al., 2012).

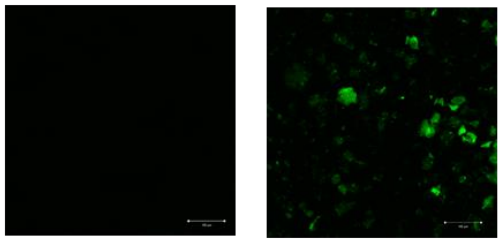
4.3.6.1. Lentiviral transduction of bm12 CD4 T cells with Bcl-6 transgene

As I have shown above that adoptive transfer of bm12 CD4 T cells into TCR $^{bd-/-}$ did not result in formation of germinal centres; I selected this model to investigate whether upregulation of Bcl-6 on donor CD4 T cells will result in development of germinal centres in TCR$^{bd-/-}$ recipients. For upregulation, I used viral transduction of donor CD4 T cells with Bcl-6 as has been described by Johnston et al (Johnston et al., 2009). The plasmid construct, vector production and bm12 CD4 T cells transduction with Bcl-6 vector are described in 2.2.11 to 2.2.14.

Bcl-6 GFP was confirmed on transduced bm12 T cells (bm12.Bcl6 CD4 T cells) under microscope while LV vector which was without Bcl-6 GFP, was used as a control vector during transduction of bm12 CD4 cells. After confirmation of Bcl-6 GFP, cells were washed and beads were removed and 2x10^6 donor bm12 CD4 T cells with Bcl-6 GFP (bm12.Bcl6) or bm12 CD4 T cells with control vector (control bm12 CD4 T cells) were injected intravenously into TCR$^{bd-/-}$ mice.

Autoantibody responses were examined by hep-2 indirect immunofluorescence and GCs were assessed by staining recipient splenic section with anti-B220, anti-CD4 and anti-GL7 antibodies. The germinal centres were significantly higher in bm12.*Bcl-6* group compared to the control group. Similarly, the autoantibodies were comparable between the two groups up to week 5 but thereafter higher in *Bcl-6*hi group compared to the control group (figure 4.22).

i.



Control bm12 CD4 T cells

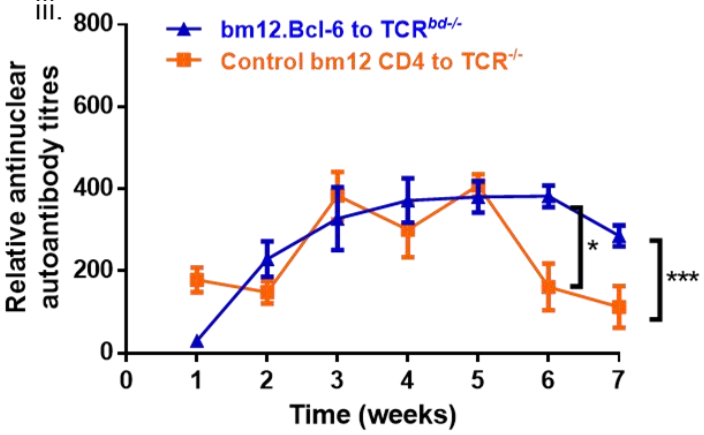
Transduced bm12 CD4 T cells with Bcl-6(GFP)

ii.

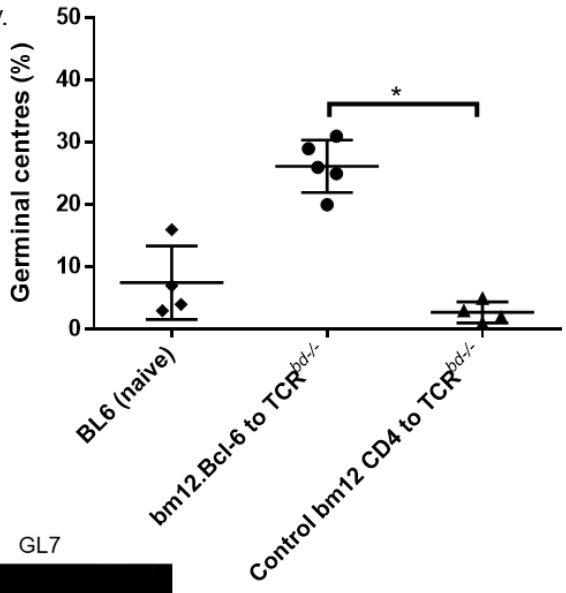
Control bm12 or bm12.Bcl-6 CD4 T cells

TCR$^{bd-/-}$ B6

iii.

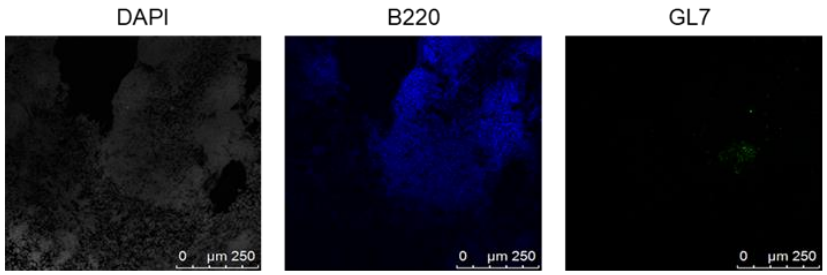


iv.



v.

Control (bm12 to TCR$^{bd-/-}$)



vi. **Bm12.bcl-6 to TCR$^{bd-/-}$**

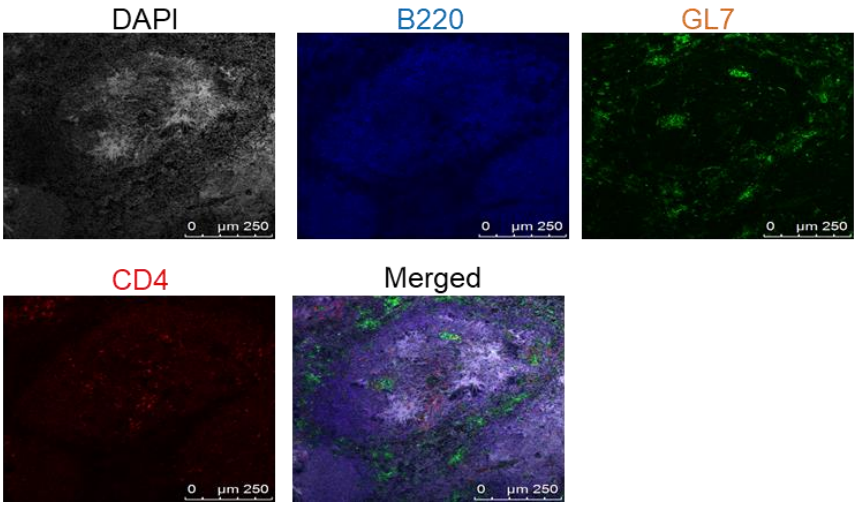


Figure 4.22. Development of long-lasting autoantibodies and GCs in TCR$^{bd-/-}$ recipients of bm12 CD4 T cells which artificially overexpressed *Bcl-6* by lentiviral transduction

i. Representative imaging depicting GFP-Bcl-6 expression in transduced bm12 CD4 T-cells and control bm12 CD4 T-cells without Bcl-6-GFP expression. Cells were cultured with Bcl-6 LV vector and control vector with stimuli and checked for fluorescence at day 4. Scale bar 100µm. Confirmation of presence of green fluorescence on donor bm12 CD4 T cells as seen under microscope showing presence of green fluorescence in bm12.Bcl-6 high group but not in the control group.

ii. Experimental model used for this experiment in which $2x10^6$ either donor bm12 CD4 T cells with high Bcl-6 or control bm12 CD4 T cells were adoptively transferred into TCR$^{bd-/-}$ recipients.

iii. Development of long lasting autoantibody in TCR$^{bd-/-}$ recipients of bm12.Bcl-6 donor CD4 T cells compared to control bm12 CD4 T cells.

iv. On quantification of germinal centres in recipient splenic sections at week 7, recipients of bm12.Bcl-6 donor CD4 T cells produced significantly higher percentages of GCs compared to control group.

v. Representative immunofluorescence images for GL7 positive staining on recipients' splenic sections; showing presence of GL7 positive B cells in follicles in bm12.Bcl-6 group but not in control group. Scale bar is 250µm.
   a. Immunofluorescence staining of recipients' splenic sections following adoptive transfer of control donor CD4 T cells demonstrating B cells did not differentiate into GCs.
   b. Immunofluorescence staining of recipients' splenic sections following adoptive transfer of donor CD4 T cells with high *Bcl-6* demonstrating presence of GL7 positive B cells in follicles confirming the presence of GCs.

Data represent mean and SD of n = 4-5 mice per group, ns=not significant, *P < 0.05, **P < 0.01 and ***P < 0.001, t-test for figure iii and Mann-Whitney test for iv.

4.3.6.2. *Affinity of GVH recognition by bm12 CD4 T cells is insufficiently strong to enable T_{FH} cell differentiation*

The second point which we wanted to assess in regards to bm12 CD4 T cells inability to become T_{FH} cell subset was to investigate whether it is the direct allo-recognition of MHC class II on recipient B cells by donor CD4 T cells which is not strong enough for them to differentiate into T_{FH} cell subset. Transfer of TCR75 CD4 T cells into the B6.Kd T cell deficient (BL6.Kd.TCR$^{bd-/-}$) mice was carried out. BL6.K^d mice express H2.K^d MHC class I antigen as a transgene (Honjo et al., 2004) and TCR75 mice recognise the dominant peptide epitope of the H2.K^d MHC class I antigen in the context of I-A^b. Because BL6.K^d mice constitutively express target I-A^b-restricted Kd peptide epitope, transfer of TCR75 T cells was expected to provoke GVH recognition and humoral autoimmunity in similar fashion to bm12 CD4 T cells (figure 4.25 i). However, we have also shown that TCR75 T cells have the ability to differentiate into T_{FH} cells in the B6 host (for example, following transplantation of BL6 recipients with a BALB/c heart graft), indicating that they recognise target K^d epitope with strong affinity (Conlon et al., 2012) (figure 4.23 ii). Thus failure of B cells to differentiate into GCs in the adoptively transferred BL6.Kd.TCR $^{bd-/-}$ recipients would support our hypothesis.

There was no detectable anti-H2-K^d IgG alloantibody, most likely because, as the H-2^d MHC antigens are membrane proteins, they provoke robust, central deletional B cell tolerance (figure 4.23 iii), however there was long lasting autoantibodies and interestingly, B cells did not differentiate into germinal centres (figure 4.23 iii, iv and v). There was late augmentation of autoantibody response probably reflecting that every TCR75 CD4 T cell, being monoclonal population of CD4 T cells, is providing help to B cells compared to polyclonal bm12 CD4 T cells.

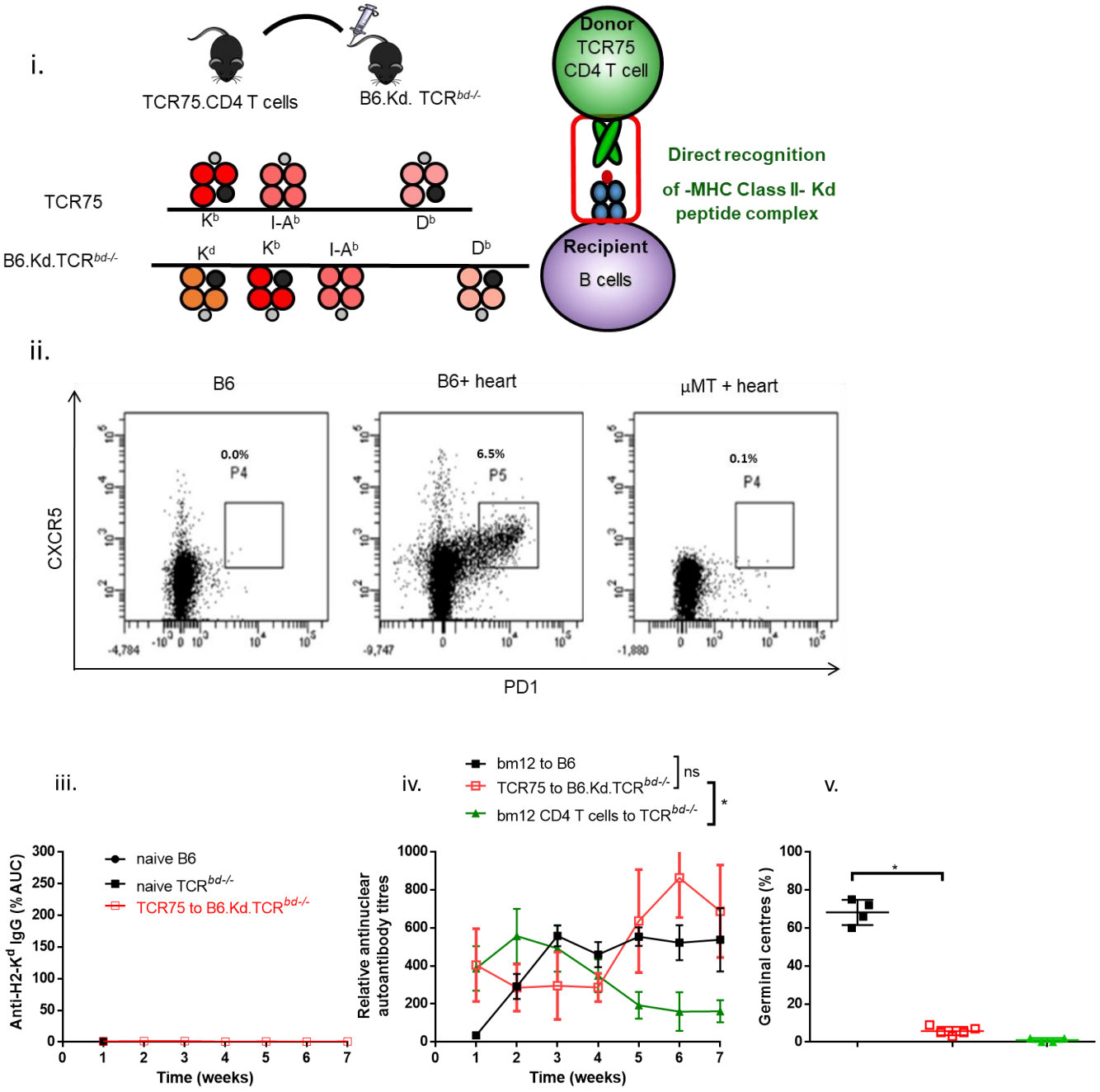

i.
TCR75.CD4 T cells
B6.Kd. TCRbd-/-
Donor
TCR75
CD4 T cell
Direct recognition
of -MHC Class II- Kd
peptide complex
TCR75
Kb
I-Ab
Db
B6.Kd.TCRbd-/-
Kd
Kb
I-Ab
Db
Recipient
B cells
ii.
B6
B6+ heart
µMT + heart
0.0%
P4
6.5%
P5
0.1%
P4
CXCR5
PD1
iii.
naive B6
naive TCRbd-/-
TCR75 to B6.Kd.TCRbd-/-
Anti-H2-Kd IgG (% AUC)
Time (weeks)
iv.
bm12 to B6
TCR75 to B6.Kd.TCRbd-/-
ns
bm12 CD4 T cells to TCRbd-/-
*
Relative antinuclear autoantibody titres
Time (weeks)
v.
Germinal centres (%)
*

Figure 4.23. Failure of B cells to differentiate into germinal centres in BL6.Kd.TCR$^{bd-/-}$ recipients following adoptive transfer of 10^3 TCR75 CD4 T cells.

i. Mice model showing adoptive transfer of TCR75 CD4 T cells from TCR75 mice into BL6.Kd.TCR$^{bd-/-}$ recipients with haplotypes for donor and recipient for MHC class I and class II and schematic pictures showing the interaction between donor TCR75 CD4 T cells with recipient B cells through direct allorecognition of K^d on MHC class I.

ii. Indirect TCR75 CD4 T cells acquiring T_{FH} cell phenotype as shown by dual positivity for CXCR5hi and PD1hi live CD T cells(Conlon et al., 2012).

iii. Serum levels of anti–H-2K^d IgG alloantibody following adoptive transfer of TCR75 CD4 T cells upto week 7.

iv. Development of autoantibodies in BL6.Kd.TCR$^{bd-/-}$ recipients following adoptive transfer of TCR75 CD4 T cells compared with bm12 to BL6 and bm12 to TCR$^{bd-/-}$ group. The autoantibodies were significantly less than TCR$^{bd-/-}$ group (bm12 to TCR$^{bd-/-}$) but comparable to WT response (bm12 to BL6).

v. Quantification of germinal centres in BL6.Kd.TCR$^{bd-/-}$ recipients of TCR75 CD4 T cells at week 7 compared with WT response and TCR$^{bd-/-}$ response. This showed that B cells did differentiate into BL6.Kd.TCR$^{bd-/-}$ and TCR $^{bd-/-}$ recipients.

Data represent mean and SD of n = 3-4 mice per group, ns=not significant, *P < 0.05, two way ANNOVA for figure iii and Mann-Whitney for iv.

4.4. Summary

In this model of graft versus host allorecognition, we have shown that

1. Adoptive transfer of cells replicated the results as those observed with heart grafts.
2. Donor CD4 T cells trigger donor induced autoimmunity in the recipients and they were found to provide T_{FH} cell function at an early stage which is transient.
3. Donor induced autoimmunity in recipients is maintained and propagated once triggered by donor CD4 T cells by a second subset of CD4 T cells which act as a T_{FH} cell for development of long term GCs. This T_{FH} cell function is provided by recruitment of recipient CD4 T cells, which act as a T_{FH} cell for development of GCs.
4. Germinal centres may be driving diversification of these humoral responses as measured by the appearance of late anti-vimentin autoantibody as a surrogate marker for humoral response diversification.
5. Monoclonal population of CD4 T cells is capable of maintaining and propagating diversification of humoral responses once initiated by donor CD4 T cells.

4.5. Discussion

Although long lasting germinal centres (figure 3.5 in Win et al paper)(Win et al., 2009) develop in our model of chronic rejection, the role of donor and recipient CD4 T cell in the development of this long lasting humoral autoimmunity was unclear. In this chapter, I have shown that donor CD4 T cells initiate and trigger the donor induced autoimmunity in the recipients but thereafter recipient CD4 T cells act as T_{FH} cells and help primary B cell follicles to differentiate into GCs for maintenance and propagation of humoral response. I further extended my findings by showing that activated recipient CD4 T cells from WT BL6 recipients are not only capable of maintaining germinal centres but they can trigger autoimmunity in naïve WT recipients as well (figure 4.16). This demonstration that one population of helper CD4 T cells initiates humoral autoimmunity in recipients, but that a second population of T_{FH} cells is required for its maintenance as a GC reaction, has important implications for how autoimmune-related phenomena manifest.

The most salient feature of this work is the presence of robust GC responses in BL6 recipients' splenic sections of bm12 CD4 T cells at week 7 (figure 4.1) and the absence of this GC autoimmune response in T cell deficient BL6 ($TCR^{bd-/-}$) recipients (figures 4.5) . Germinal centre response was restored when the T cell deficient recipients were reconstituted with host BL6 CD4 T cells simultaneously (figure 4.7). The criticism regarding $TCR^{bd-/-}$ recipients was that B cells in these animals might not be developing normally in the absence of host T cells, hence B cells in the $TCR^{bd-/-}$recipients did not had the ability to differentiate into secondary follicles i.e. germinal centres. In this respect although haematoxylin and eosin staining of naive $TCR^{bd-/-}$animals splenic sections did not show any specific follicular architecture, reconstitution with WT BL6 CD4 T cells restored their follicular architecture and simultaneous administration of bm12 CD4 T cells and WT BL6 CD4 T cells resulted in development of GCs (figure 4.4), thus suggesting critical role of recipient CD4 T cells. However, absence of late GCs when SAP signalling was inhibited in recipient CD4 T cells (*Sh2d1a*$^{-/-}$) and restoration of late GCs when SAP signalling was inhibited on donor bm12 CD4 T cells (*Sh2d1a*$^{-/-}$) by using SAP^{-ve} BL6 recipients and SAP^{-ve}.bm12 donor CD4 T cells respectively confirmed this critical role of recipient CD4 T cells (figure 4.14 and 4.15). *Sh2d1a*$^{-/-}$ mice are thought to have essentially normal CD4 T and B cells with relatively narrow defects in NK T cells (Czar et al., 2001). Furthermore, the ability of activated

recipient CD4 T cells in triggering autoantibodies in naïve WT BL6 confirms this role that they are not capable only in initiating humoral autoimmunity in recipients but they can propagate this through providing help to B cells for development of GCs (figure 4.16).

Although there was early GCs in SAP^{-ve} recipients of bm12 CD4 T cells, it did not sustain for long term (figure 4.14), this is in consistent with presence of donor bm12 CD4 T cells in CD45.1.BL6 recipients at day 16 and day 30 (figure 4.11 and 4.12). Furthermore, our initial experiments with adoptive transfer also confirmed the presence of a population of donor bm12 CD4 T cells acquiring T_{FH} cell phenotype at day 23 (Qureshi et al., 2019). From this it is possible that donor CD4 T cells are providing initial transient T_{FH} cell function at an early time point but at late time point it is the recipients CD4 T cells which are providing the critical T_{FH} cell function for long term humoral autoimmunity. This raised many unanswered questions like why donor bm12 CD4 T cells do not acquire T_{FH} cell function and what is the nature of antigen recognition by the recipient T_{FH} cell population.

The potential reasons for failure of bm12 CD4 T cells to act as T_{FH} cells could be due to their short life span in the host / exhaustion / down regulation of Bcl-6 (master regulator) on donor CD4 T cells because of their interaction on MHC class II on recipient DCs or weaker interaction with MHC II-peptide complex which is not strong enough to sustain the T_{FH} cell function.

In this respect, inability of TCR75 CD4 T cells to acquire T_{FH} cell function when interacting with peptide-MHC class II complex in BL6.Kd.TCR$^{bd-/-}$ in a direct allorecognition fashion, (figure 4.24) especially with our previous work in which we have shown that these TCR75 CD4 T cells acquire T_{FH} cell function when recognising dominant peptide in the context of I-A^b through indirect allorecognition (Conlon et al., 2012), support the concept that CD4 T cells acquiring T_{FH} cell function depends on their pathway of allorecognition (Macdonald et al., 2009). Recipient CD4 T cells might be acquiring T_{FH} cell function because of the unusual nature of allorecognition in this model. Interaction of donor CD4 T cells with recipient MHC-peptide complex activates multiple clones of CD4 T cells (precursor frequency of ~5 – 10%) (Busser et al., 2003), but this interaction may be of low affinity and we know that only those CD4 T cells which interact with MHC-peptide complex with highest affinity will differentiate into T_{FH} cells (Fazilleau et al., 2009). Furthermore, due to this unusual form of direct allorecognition, TCR75 CD4 T cells may not maintain their T_{FH} function and GC maintenance

requires renewal of the T_{FH} cell by continual differentiation of secondary subset of CD4 T cells during the course of a GC response. In support, recent work by Shulman has demonstrated that unlike GC B cells, which are clonally restricted, T_{FH} cells emigrate from the follicles and invade the neighbouring follicles. These newly emigrated T_{FH} cells then contribute to B cell selection and augment ongoing GC responses (Shulman et al., 2013).

The other possibility of down regulation of Bcl-6 in donor bm12 CD4 T cells due to successive interaction with DCs may be the limiting factor for them to acquire T_{FH} cell function. In support, artificial overexpression of Bcl-6 directs them to acquire T_{FH} cell function and they help B cells to differentiate into GCs (figure 4.23) which is in consistent with the previous work where it has been shown that artificial overexpression of Bcl-6 on CD4 T cells help them to differentiate into T_{FH} cells (Johnston et al., 2009).

Demonstration of a monoclonal population of CD4 T cells which are capable of driving and maintaining humoral response diversification in $TCR^{bd-/-}$ recipients of bm12Kd and TCR75 CD4 T cells (figure 4.22), supports that TCR75 T_{FH} CD4 T cells might be providing help to autoreactive GC B cells for development of long term donor induced autoimmunity. As we know that bm12.Kd cells get killed within one week of their adoptive transfer and thereafter it is the TCR75 CD4 T cells which would be providing help for development of GCs (Harper et al., 2016). However we also know that TCR75 CD4 T cells differentiate into T_{FH} cell while recognising dominant peptide K^d on I-A^b and result in development of only anti-Kd alloantibody (Conlon et al., 2012), and potentially these GC are exclusively Kd specific. But apart from long term alloantibody, there is a long lasting autoantibody response as well in recipient sera with development of late autoimmune response to vimentin in this group compared to $TCR^{bd-/-}$ recipients challenged with bm12.Kd cells alone. This may propose that TCR75 T_{FH} cells would be providing help to autoreactive GC B cells by providing help to the already existing autoreactive GC B cells. This seems true especially in the light of early GCs in SAP^{-ve}.BL6 recipients of bm12 CD4 T cells. Furthermore, this set of experiment also demonstrates that B cells are capable of driving the epitope diversification of humoral response which is in consistent with Mamula et al work (Mamula, 1998; Mamula and Janeway, 1993). However, it contradicts to Mamula's work in suggesting that B cell epitope diversification is possible in the absence of T cell epitope diversification as there was only monoclonal population of CD4 T cells which was capable of driving the diversification of

humoral repose in case of GVH allorecognition. However, it is important to examine that whether TCR75 CD4 T cells would be capable of triggering autoimmune humoral response and diversification of that humoral response in the absence of GVH allorecognition by assessing humoral responses in B6.TCR$^{bd-/-}$ recipients of B6.Kd cells which have simultaneously received TCR75 CD4 T cells.

This raised multiple interesting questions that how TCR75 CD4 T cells (K^d specific) are providing help to the development of long lasting autoantibody response and whether autoimmunity is the result of GC response or EF response. It seems likely that diversification of humoral response is the result of GC response as there were no GCs and late anti-vimentin autoantibody when SAP^{-ve}TCR75 CD4 T cells were transferred into TCR$^{bd-/-}$ recipients (figure 4.22). The possible explanation may be that during affinity maturation, K^d specific B cells may pick up autoantigens and receive unlinked help from TCR75 CD4 T cells to develop ASC for autoantibodies which remains unchecked due to unavailability of T follicular regulatory cell (T_{FR}) control. However, this needs further work to confirm that GCs are purely K^d specific and to identify the mutations at BCR which leads to the development of autoimmune response. Furthermore, more work is required to identify that whether the adoptively transferred subset of TCR75 CD4 T cells may develop into T_{FH} cell only or T_{FR} cell as well.

The differentiation of recipient T_{FH} cells following adoptive transfer of bm12 CD4 T cells is presumably driven by antigen presentation by B cells that have been activated following interaction with donor CD4 T cells. B cells are then capable of 'soliciting' their own help (Stockinger et al., 1996), and hence, it is likely that recipient CD4 T cells are responding to self-restricted peptide autoantigen that is presented by the activated B cell.

In this model, the demonstration of a late anti-vimentin response in recipients' groups who developed germinal centres in their splenic sections may suggest that diversification of humoral autoimmune response is potentially the result of germinal centres. In the process of somatic hypermutation and affinity maturation it is possible for GC B cells to encompass additional autoantigens, including pathogenic ones, and thus lead to the development of pathologic disease in the recipients like SLE or RA. The autoantigen availability during GC response may be the result of apoptosis during affinity maturation. Although I chose to study the anti-vimentin response as a surrogate marker for diversification of humoral

response due to previously-reported association with rheumatoid arthritis, SLE (Davidson, 2014) and in allograft vasculopathy (Mahesh et al., 2007; Rose, 2013), despite the development of late anti-vimentin in recipients, it is surprising that autoimmune disease manifestations were not observed routinely in our recipients' native organs (Harper et al., 2016; Motallebzadeh et al., 2012; Win et al., 2009), particularly when one considers that administration of bm12 splenocytes into BL6 model is used as a model for SLE (Eisenberg and Via, 2012). We do see occasional skin and gut manifestations, consistent with autoimmune disease, in long-term surviving recipients of bm12 CD4 T cells (> 6 months), however comparing this work with the previous work, the difference of appearance of disease may be related to number and type of cells transferred into recipients.

In conclusion, I have shown that donor CD4 T cells initiate donor induced autoimmunity in the recipients and thereafter recipient CD4 T cells act as T_{FH} cell and provide help to autoreactive B cells to differentiate into GC which in turn diversify humoral autoimmune response. It also provides an insight into the underlying pathogenesis of autoimmune disease that at the time of disease manifestations, the trigger for the autoimmunity like viral challenge may not be present but there may be a different factor which may be maintaining the disease process. Like in this scenario, it is the recipient T_{FH} cells which are maintaining and propagating the donor induced auto-immunity in the recipient and developing selective strategies to target the pathogenic population of T_{FH} cells may be a future avenue to deal with different autoimmune diseases.

Chapter 5

Germinal centre autoantibody responses contribute to progression of allograft vasculopathy

5.1. Introduction

Although allograft rejection is generally considered to be mediated by adaptive immune recognition of graft alloantigens, an association of host autoimmunity with chronic rejection of organs has been increasingly reported (Dave and Bayless, 2014; Dragun et al., 2013; Dragun et al., 2016; Fedoseyeva et al., 2002; Fedoseyeva et al., 1996; Fedoseyeva et al., 1999; Haque et al., 2002; Mahesh et al., 2007; Zhang and Reed, 2016), particularly following heart and lung transplantation. These autoimmune responses were directed against several tissue specific self-proteins and appear to be the direct result of transplant, however the mechanisms that lead to their development and contribution to graft rejection remain poorly understood. I have discussed in detail the role of autoimmunity in chronic rejection in section 1.5. Briefly, the strongest clinical evidence for an effector role of autoimmunity contributing to chronic rejection of organs is from lung transplant recipients where T cell de novo autoimmunity directed against collagen type V protein has been shown contributing to progression of bronchiolitis obliterans syndrome in recipients (Burlingham et al., 2007). Similarly in heart transplant recipients' antibodies against cardiac myosin (CM) or detection of CM peptide-reactive T cells was highly and independently indicative of allograft vasculopathy (Kalache et al., 2011). Furthermore, in kidney transplant recipients, antibodies to collagen-IV and fibronectin have been shown contributing to transplant glomerulopathy (TG) (Angaswamy et al., 2014). Nevertheless, several key questions relating to transplant associated autoimmunity (TAA) remain unanswered. It is not known whether autoimmunity contributes to chronic rejection independently or whether it is augmenting conventional alloimmune response directed against the allograft. In Angaswamy et al study, development of antibodies to self-antigens (Ags) like fibronectin and collagen IV was thought to augment the deleterious effects of DSA in TG recipients (Angaswamy et al., 2014). In another study in lung transplant patients, recipients with pre-existing autoantibodies to self-antigens were found to be at an increased risk for development of primary graft dysfunction and chronic rejection (Tiriveedhi et al., 2013).

There are only few rodent studies which suggest an effector role for autoimmune responses in chronic graft rejection. Fedoseyeva et al (Fedoseyeva et al., 2002; Fedoseyeva et al., 1999) showed that modulation of T cell response to cardiac myosin (CM) can result either in accelerated rejection (Fedoseyeva et al., 1999), or indefinite heart graft survival in the

absence of immunosuppression (Fedoseyeva et al., 2002). Their experimental work suggested that immunity to CM detected in heart-grafted mice is consistently mediated by CD4 T cells releasing Th-1 cytokines (IL-2, IFN-ɣ), but not type 2 cytokines. Sensitization of recipient mice to CM before transplantation induces a potent proinflammatory Th1 anti-CM response which results in accelerated rejection of donor graft.

Similarly, in another rodent study, collagen V specific CD4 T cell lines were isolated from lung allograft recipients which proliferated in response to collagen V but did not induce any pathology in lungs of normal rats. These cell lines were $CD4^{+ve}$ and $CD25^{-ve}$ but produced IFN-gamma in response to collagen V. Adoptive transfer of one of these cell line abrogated the established transplant tolerance in lung allografts and produced severe inflammatory changes in iso-grafts (Haque et al., 2002). Thus, their work demonstrated that lung allograft rejection involves both allo- and autoimmune responses, and graft destruction that occurs during the rejection response may expose allograft-infiltrating T cells to potentially antigenic epitopes in collagen (V). However, these studies have focused only on T lymphocyte autoimmune responses; but not how they develop in post-transplant recipients and contribute to graft rejection.

Most of the work on how these autoimmune responses develop comes from conventional immunology looking at various autoimmune diseases like SLE and RA. In this regard, although humoral autoimmune disease was traditionally thought to be germinal centre (GC) mediated (Brink, 2014; Vinuesa et al., 2009), extrafollicular (EF) responses can also result in affinity matured autoantibody production (William et al., 2002). However, it is not clear how autoantibodies form in transplant recipients whether they originate from GCs or EF or both. Nevertheless, recent work in conventional immunology has shown that GC autoimmune responses require help from T_{FH} cells (Linterman et al., 2009; Vinuesa et al., 2016) and it raises the possibility of developing selective strategies that target this subset, in anticipation of prolonging allograft survival in recipients.

In the previous chapter, I have shown that in bm12 to BL6 heart transplant model, long lasting autoantibodies are the result of germinal centres (figure 3.5) and T_{FH} cells were identified based on their homing to GC B cells (figure 3.6) and phenotypic markers (figure 3.8) (chapter 3). Furthermore, adoptive transfer experiments (chapter 4) demonstrated that donor CD4 T cells initiate and trigger donor induced autoimmunity in recipients but

thereafter, host T_{FH} cells maintain and propagate this autoimmunity through germinal centre formation. In this chapter we will extend our findings of adoptive transfer experiments into murine heart allograft model of chronic rejection to identify the role of donor and recipient CD4 T cells in TAA and its contribution to the development of allograft vasculopathy. This will also help us to identify which of them is acting as a T_{FH} cell. Identification of T_{FH} cell will help us to develop future strategies to selectively block T_{FH} cells to inhibit GC autoimmune response in order to prolong allograft survival. Of note, the experimental details, results, discussion and future implications written in this chapter has already been published in my papers (Qureshi et al., 2019; Qureshi et al., 2018).

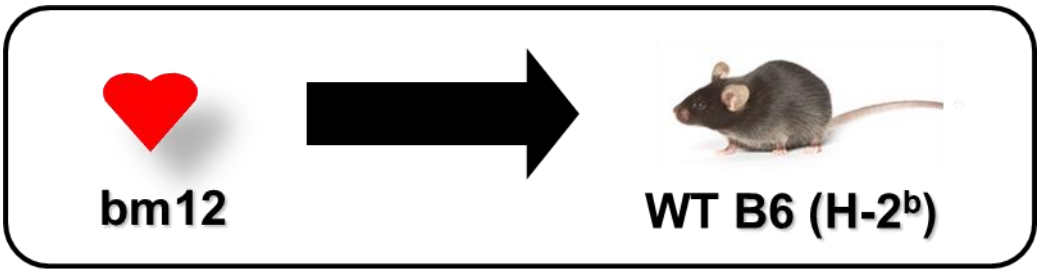
5.2. Aims

The aims of this chapter are:

1. To identify the relative contribution of donor and recipient CD4 T cells in the development of transplant associated auto-immunity.
2. To investigate whether GC autoimmunity contributes to progression of allograft vasculopathy independent of alloimmune responses.

5.3. Experimental model

In order to examine the role of donor and recipient CD4 T cells in the development of TAA, I used wild- type (WT) mice and T cell deficient mice (that lack all TCR-αβ and TCR-γδ CD4 and CD8 T cells) ($TCR^{bd-/-}$) as recipients of either CD4 T cell-replete or CD4 T cell-depleted bm12 heart allografts. Furthermore, in order to assess whether the observed difference in outcomes between WT and $TCR^{bd-/-}$ recipient was related to deficiency in specific T cells population, in certain experiments, $TCR^{bd-/-}$ recipients were reconstituted with purified syngeneic WT BL6 CD4 T cells on the day of transplant (figure 5.1).



	bm12 Donor	BL6BL Recipient	CD4 T cell transfer
WT	WT	WT	–
Rc_{CD4}	CD4 T cell depleted	WT	–
Dn_{CD4}	WT	TCR $bd^{-/-}$	–
$Dn.Rc_{CD4}$	WT	TCR $bd^{-/-}$	+BL6 CD4

Figure 5.1. Experimental model showing WT, Rc_{CD4}, Dn_{CD4}, $Dn.Rc_{CD4}$ groups used to identify the role of donor and recipient CD4 T cells.

Antinuclear autoantibodies were assessed by indirect immunofluorescence in recipient sera (section 2.2.4), germinal centre activity was examined by staining recipients splenic cryo sections for germinal centre makers (section 2.2.7.3), graft survival was assessed by manual palpation of donor hearts in the recipients (section 2.1.2.1) and allograft vasculopathy was calculated morphometrically by measuring the mean luminal stenosis of intra myocardial vessels of the explanted hearts (section 2.2.7.5) as described in methods. GCs were quantified at day 50 or week 7 unless stated otherwise. Allograft vasculopathy was calculated at day 50 or week 7 unless stated otherwise. For autoimmune responses to vimentin, anti-vimentin ELISA was performed on recipient sera at week 7 and week 15 for development of late anti-vimentin autoantibody as described in section 2.2.5.3.

5.4. Results

5.4.1. Both donor and recipient CD4 T cells contribute to progression of allograft vasculopathy

As expected from my previous chapter work, class-switched anti-nuclear autoantibodies form when CD4 T cell help was provided exclusively by the donor CD4 T cell (bm12 to TCR$^{bd-/-}$), these autoantibody titres were comparable with WT response (bm12 to BL6) (figure 5.2 a). However, there was no autoantibody when B cell help was only provided by the recipient CD4 T-cells (CD4 depleted bm12 to WT.BL6) (figure 5.2 a). Interestingly, CD4 T-cell replete recipients of CD4 T-cell replete donors developed autoantibodies which were comparable to WT response. Despite the development of autoantibodies in TCR$^{bd-/-}$ recipients of CD4 T cell-replete bm12 heart allografts; the donor grafts did not develop AV. However, AV only developed when both donor and recipient were CD4 T cell replete (figure 5.2b), resulting in allograft rejection (figure 5.2 c). This demonstrated two things; firstly that both donor and recipient CD4 T cells are required for allograft rejection and secondly autoantibodies seem to be playing a role in rejection as there was no AV in CD4 T cell depleted bm12 donor allografts when transplanted into WT BL6 recipients (figure 5.2a) (Qureshi et al., 2018).

Although our previous studies have suggested a role of autoantibody in graft rejection (Motallebzadeh et al., 2012; Win et al., 2009); in order to find out a definite contribution of autoantibody in chronic rejection of allograft in this model, I hypothesised that augmented autoantibody response in the recipients will result in accelerated rejection of donor bm12 allografts compared to WT rejection kinetics. In order to do this, I primed bm12 hearts by transplanting WT BL6 skin grafts to bm12 recipients to get bm12 heart grafts that contained memory CD4 T cells with specificity for the WT BL6 recipients. Weekly tail bleeds were carried out from the bm12 recipients and flow cytometry was performed on peripheral blood lymphocytes (PBLs) to check for memory CD4 T cells phenotype by staining with anti-CD44 and anti-CD62L antibodies as described in methods (methods section 2.2.1.3 and 2.2.3.2). Flow cytometry of PBLs demonstrated memory phenotype of CD4 T cells after week 4. At week 7, bm12 recipients of BL6 skin grafts were sacrificed; spleens and hearts were harvested from the recipients. Bm12 primed hearts were transplanted into WT BL6 recipients and spleens were examined to confirm the presence of memory CD4 T cells (figure 5.3a). Humoral response was examined by autoantibody responses in recipients' sera and germinal centres by quantification of GL7 positive B cells. Augmented autoantibody

response were observed in the primed group compared to WT (figure 5.3b), in keeping with the presence of germinal centres in recipient splenic section (figure 5.3c) and rapid rejection of the donor heart allograft was observed with AV (figure 5.3d and e) (Qureshi et al., 2019; Qureshi et al., 2018).

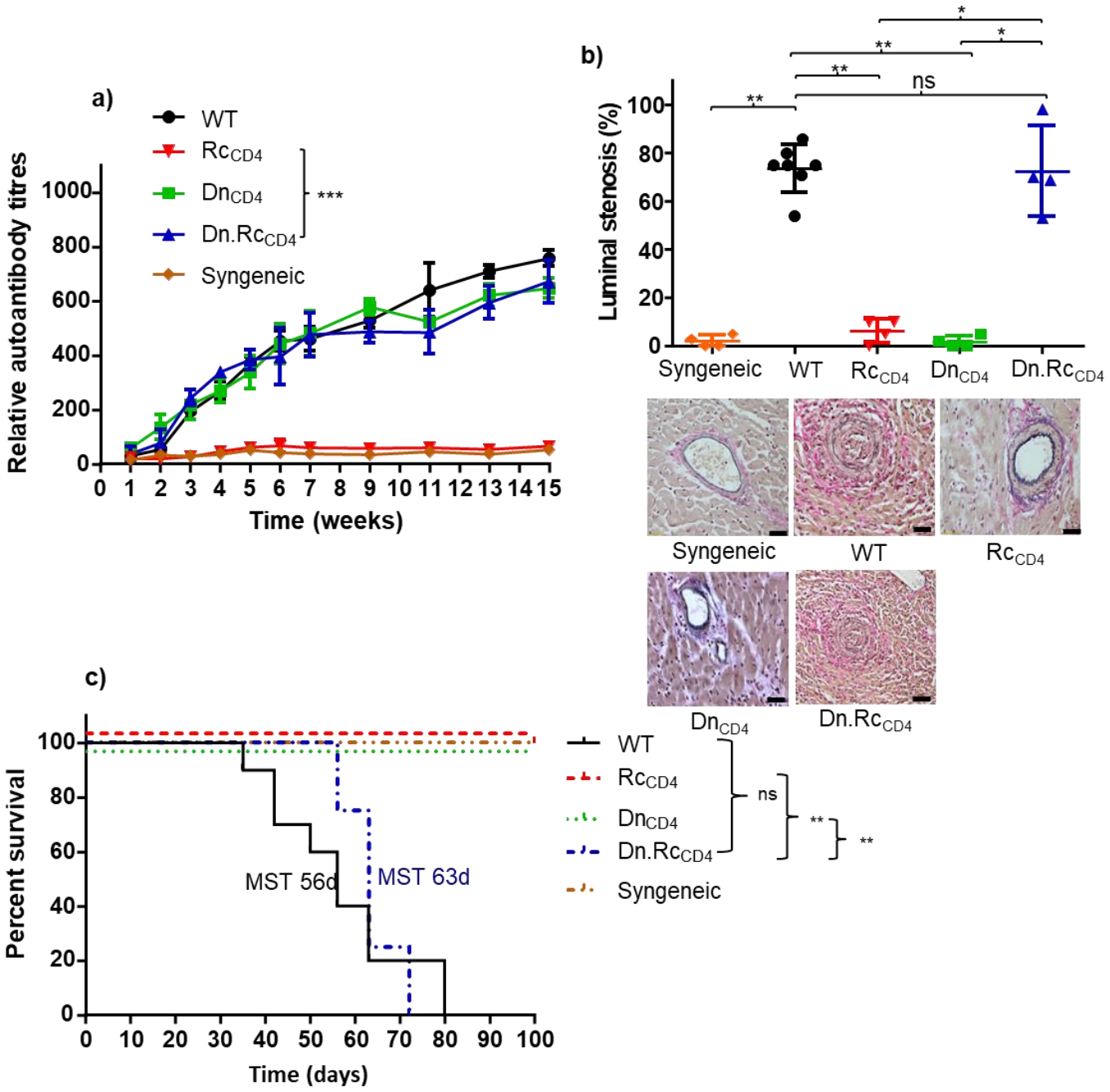

a)
WT
Rc$_{CD4}$
Dn$_{CD4}$
Dn.Rc$_{CD4}$
Syngeneic
***
Relative autoantibody titres
Time (weeks)
b)
Luminal stenosis (%)
ns
Syngeneic
WT
Rc$_{CD4}$
Dn$_{CD4}$
Dn.Rc$_{CD4}$
c)
Percent survival
MST 56d
MST 63d
Time (days)
ns
**

Figure 5.2. Donor and recipient CD4 T cell populations are required for autoantibody-mediated allograft vasculopathy

a). Autoantibody development was dependent upon transfer of donor bm12 CD4 T cells, but independent of recipient CD4 T cells. Autoantibody responses in BL6 recipients of syngeneic BL6 heart allografts are also shown.

b). At explant, the severity of allograft vasculopathy was determined morphometrically and expressed as percentage luminal stenosis (histogram), with representative photomicrographs of elastin van Gieson stained paraffin sections, depicting typical fibro-proliferative arterial intimal thickening observed in rejecting heart allografts (scale bars 50µm). Severe vasculopathy was only observed in groups in which donor and recipient CD4 T cell subsets were present.

c) Kaplan-Meier curves depicting allograft survival.

Data represent mean and SD of n = 4-10 mice per group, with discrete data-points in **(b)** depicting individual animals. ns – not significant: *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$: 2-way ANOVA in (b), Mann-Whitney test in (b), log rank test in (c).

Figure 5.3

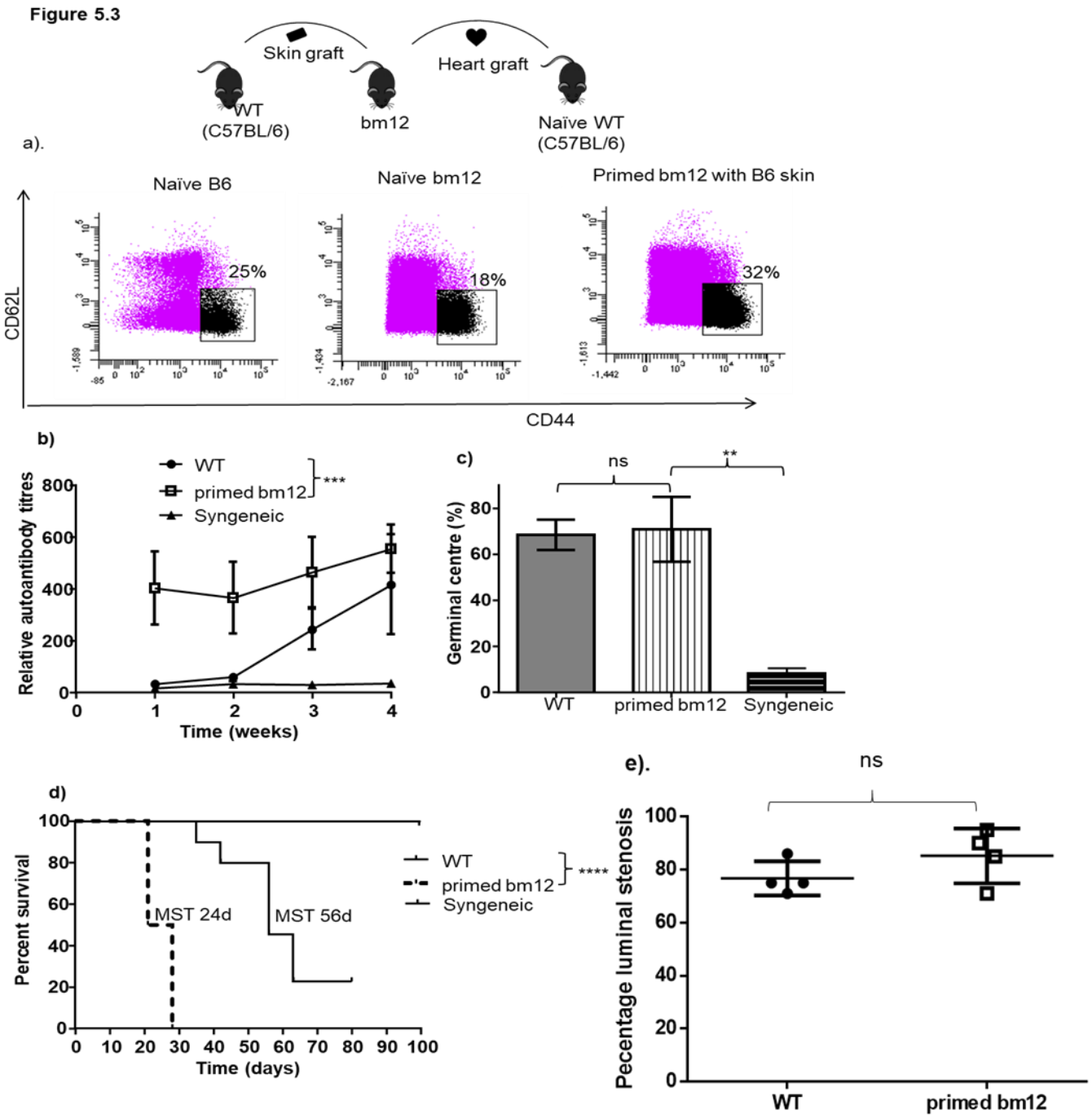

a).
Skin graft
Heart graft
WT
(C57BL/6)
bm12
Naïve WT
(C57BL/6)
Naïve B6
Naïve bm12
Primed bm12 with B6 skin
CD62L
CD44
25%
18%
32%
b)
WT
primed bm12
Syngeneic
***
Relative autoantibody titres
Time (weeks)
c)
ns
**
Germinal centre (%)
WT
primed bm12
Syngeneic
d)
Percent survival
Time (days)
MST 24d
MST 56d
WT
primed bm12
Syngeneic
****
e).
ns
Pecentage luminal stenosis
WT
primed bm12

Figure 5.3: Augmented anti-nuclear autoantibody response and accelerated heart allograft rejection following transplantation from donors primed against recipient

- **(a)** WT (BL6) mice were transplanted with hearts from bm12 donors sensitised to recipients by challenge with WT (BL6) skin grafts 7 weeks previously (primed bm12). Control recipient BL6 mice received heart allografts from unmodified bm12 donors (WT). Memory CD4 T cells were identified with live, CD4 T cells, having $D62^{lo}$ and $CD44^{hi}$ markers on flow cytometry.
- **(b)** Anti-nuclear autoantibody titres following transplantation.
- **(c)** Recipient's splenic germinal centre activity at week 4 post transplantation. (percentage of secondary follicles to total number of follicles).
- **(d)** Kaplan-Meier curves depicting allograft survival.
- **(e)** Donor allograft vasculopathy in primed bm12 hearts at their explants compared to WT donors AV.

Data represent mean and SD of n = 4-10 mice per group. ns – not significant: $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$: 2-way ANOVA in (b), Mann-Whitney test in (c, e) and log rank test in (d).

5.4.2. Host T_{FH} cells are required for maintenance of germinal centre autoantibody response

Because the bm12 allografts did not demonstrate allograft vasculopathy on explantation from $TCR^{bd-/-}$ recipients despite the presence of autoantibody response in the recipients' sera, we hypothesised that the host CD4 T cells may be providing critical role of T_{FH} cell function for the development of germinal centre response in the WT BL6 recipients and those GC mediated humoral responses may be contributing to the rejection of donor allografts. In order to investigate this, germinal centre activity was assessed in recipients' splenic cryo sections at week 7 post transplantation. Splenic sections were stained as described in methods (2.2.7.3) and quantified as described in methods (2.2.7.5).

On staining, splenic GC activity was not detectable in $TCR^{bd-/-}$ recipients of WT bm12 heart allografts or in WT BL6 recipients of CD4 T cell depleted donors, but was readily evident in WT BL6 recipients of WT bm12 heart grafts or in $TCR^{bd-/-}$ recipient mice that had been reconstituted with recipient WT BL6 CD4 T cells, with the majority of B cell follicles exhibiting GL7 positive GCs (figure 5.4a). This demonstrated that both donor and host CD4 T cells are required for the development of GCs. The next question was whether it is donor or recipient or both CD4 T cell populations which are providing the critical T_{FH} cell function.

In order to investigate this, I used confocal immunofluorescence microscopy to locate the presence of CD4 T cells within the GC follicles which would be consistent with the T_{FH} cell subset (Yu and Vinuesa, 2010). On staining, a population of CD4 T cells was identified in BL6 recipients of bm12 heart allografts within splenic GCs (figure 5.4b). However both donor and recipient cells were CD45.2 positive, hence I was unable to tease out whether the CD4 T cells were of donor or recipient origin. In order to investigate this, I challenged CD45.1 BL6 mice with congenic CD45.2 bm12 allografts and confocal imaging was performed on recipient splenic sections seven weeks post transplantation. Although CD4 T cells were identified within the GCs, donor CD4 T cells (CD45.2 positive) were not detectable (figure 5.4b, 3rd row). This suggested that potentially the follicular helper CD4 T cells were predominantly of recipient origin. However, it was not possible to confirm this with CD45.1 staining because of simultaneous binding of anti-CD45.1 antibody to surrounding endogenous (recipient) CD45.1 positive B cells (figure 5.4b, 4^{th} row).

In order to overcome this caveat, GC splenic responses were assessed seven weeks after transplantation of CD45.2 bm12 heart grafts into CD45.2.TCR$^{bd-/-}$ BL6 recipients that had been simultaneously reconstituted with CD45.1.BL6 CD4 T cells at the time of transplantation. Using this approach, a population of the transferred congenically labelled (CD45.1^{+ve}) BL6 CD4 T cells were readily evident within the GC B cells (figure 5.4c)(Qureshi et al., 2018).

Figure 5.4

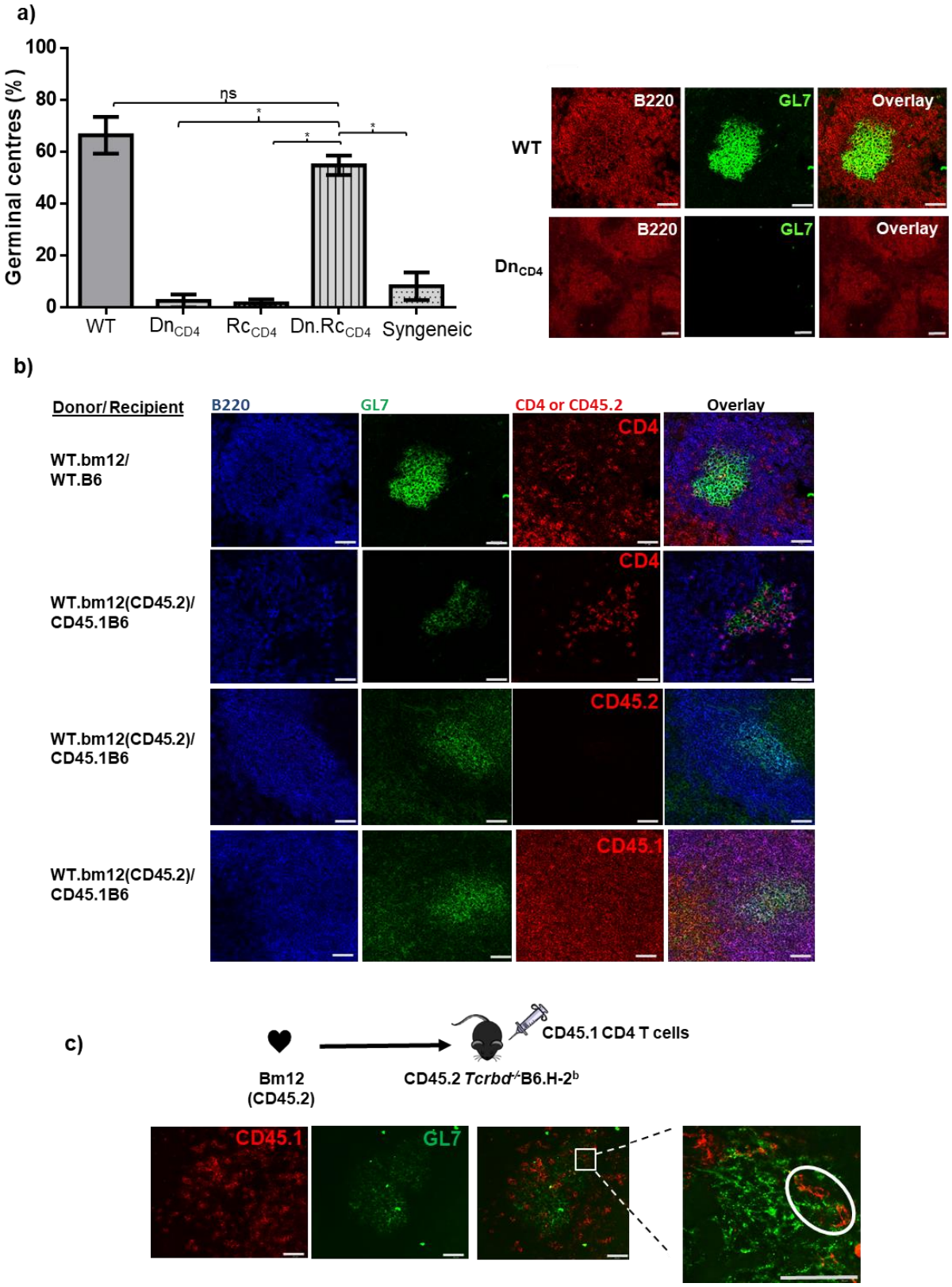

a)
Germinal centres (%)
100
80
60
40
20
0
ns
*
*
*
WT
Dn$_{CD4}$
Rc$_{CD4}$
Dn.Rc$_{CD4}$
Syngeneic
B220
GL7
Overlay
WT
Dn$_{CD4}$
b)
Donor/ Recipient
B220
GL7
CD4 or CD45.2
Overlay
WT.bm12/
WT.B6
CD4
WT.bm12(CD45.2)/
CD45.1B6
CD4
WT.bm12(CD45.2)/
CD45.1B6
CD45.2
WT.bm12(CD45.2)/
CD45.1B6
CD45.1
c)
CD45.1 CD4 T cells
Bm12
(CD45.2)
CD45.2 Tcrbd-/-B6.H-2b
CD45.1
GL7

Figure 5.4: Recipient T follicular helper CD4 T cells are required for maintaining recipient germinal centre autoantibody responses

a. Frequency of splenic GCs, expressed as a proportion of total B cell follicles (histogram), in the various recipient groups as depicted in figure 5.2. Images depict representative confocal images of $GL7^{pos}$ GCs within recipient splenic B cell follicles for the wild-type donor / recipient combination (top row) and for wild-type bm12 hearts transplanted into *Tcrbd*$^{-/-}$ BL6 recipients (bottom row). *$P < 0.05$ Mann-Whitney test (n=5).

b. Confocal immunohistochemistry of splenic sections seven weeks after transplantation of wild-type (WT) BL6 mice with WT bm12 heart allografts (top row) confirmed the presence of T cells (red) within GC foci ($GL7^{pos}$:green) in B cell follicle (blue). Following transplantation of CD45.2 bm12 heart allografts into congenic CD45.1 BL6 recipients, CD4 T cells within secondary GC follicles were again readily evident (2nd row). However, congenically labelled (CD45.2) donor cells were not detectable in the B cell follicle (3rd row). Due to the simultaneous presence of CD45.1 on B cells in CD45.1 BL6 recipients, the staining for $CD45.1^{+ve}$ CD4 T cells is not conclusive (4th row).

c. Seven weeks after transplantation of CD45.2 bm12 hearts into CD45.2 *Trcbd*$^{-/-}$ BL6 mice CD4 T cells, a population of congenic (CD45.1) *recipient* BL6 CD4 T cells (transferred at transplantation) was readily evident within the GC (far right – expanded image with CD45.1 CD4 T cell population circled.

Data are representative of 4 mice per group (a) and of two independent experiments (b & c). Image scale bars; 40μm for figure a, b and c.

5.4.3. Inhibition of SAP signalling on recipient CD4 T cells prolongs graft survival

In the previous chapter, I have shown that inhibition of SAP signalling in recipient CD4 T cells prevents development of long term GCs in SAP^{-ve}.BL6 recipients of bm12 CD4 T. I extended these findings to transplant settings to answer two questions:

- Firstly, to address the role of recipient CD4 T cells as T_{FH} cells in maintaining GC humoral autoimmunity following transplantation,
- And secondly, to assess that whether blocking T_{FH} cells in donors or recipients prolongs allograft survival?

In order to investigate this, WT bm12 or *Sh2d1a*$^{-/-}$.bm12 (SAP^{-ve}. bm12) donor heart allografts were transplanted into *Sh2d1a*$^{-/-}$BL6 (SAP^{-ve}.BL6) or WT BL6 recipients. Recipient sera were assessed for autoantibody response, splenic sections were looked for germinal centres and donor grafts were assessed for development of AV.

As expected from my adoptive transfer work (previous chapter); transplantation into SAP^{-ve}.BL6 recipients resulted in antinuclear autoantibody responses, however autoantibodies were either equal or more than those observed in WT BL6 recipients (figure 5.5a). Interestingly, splenic germinal centre activity was not above the base line when assessed seven weeks after transplantation (figure 5.5b). Importantly, in the absence of late germinal centre activity, heart grafts survival was prolonged (figure 5.5c) and allograft vasculopathy was minimal (figure 5.5d).

Although we have shown previously that there was no autoantibody in B cell deficient recipients (µMT) of bm12 heart allografts and AV was attenuated in donor allografts, AV was measured by allograft severity score (Win et al., 2009) which is different from the degree of luminal stenosis as measured by morphometric analysis (method section 2.2.7.5) in my work. Hence, bm12 donors were transplanted into B cell deficient recipients (µMT) and AV was measured again in this work to rule out any contribution from cellular arm of adaptive alloimmunity. On calculation of percentage luminal stenosis in donor allografts, allograft vasculopathy of bm12 donor allografts in SAP^{-ve}.BL6 recipients was similar in severity as that observed in µMT recipients (figure 5.5d).

This demonstrated that blocking SAP signalling in recipient CD4 T cells resulted in failure of B cells to differentiate into GCs and prolonged allograft survival with minimal AV; hence

suggesting that recipient CD4 T cell are acting as T_{FH} cell. Then I sought to investigate whether donor CD4 T cells are acting as T_{FH} cell as well by transplanting SAP^{-ve}.bm12 heart grafts into WT BL6 recipients.

On analysis, WT BL6 recipients of SAP^{-ve}.bm12 heart grafts developed GC autoantibody responses (figure 5.5 a and b) that were comparable to the responses observed in WT. SAP^{-ve}.bm12 allografts were rejected at the same tempo as that observed in WT bm12 heart grafts (figure 5.5c). The severity of AV was also similar between SAP^{-ve} bm12 and WT groups (figure 5.5d). This demonstrated that donor CD T cells can interact with recipient B cells even in the absence of SAP signalling in donor CD4 T cells and long lived GCs still form in splenic follicles in the WT BL6 recipients, which explains that T_{FH} cell function is being provided by the recipient CD4 T cells which were SAP competent in the recipients (Qureshi et al., 2018).

Figure 5.5

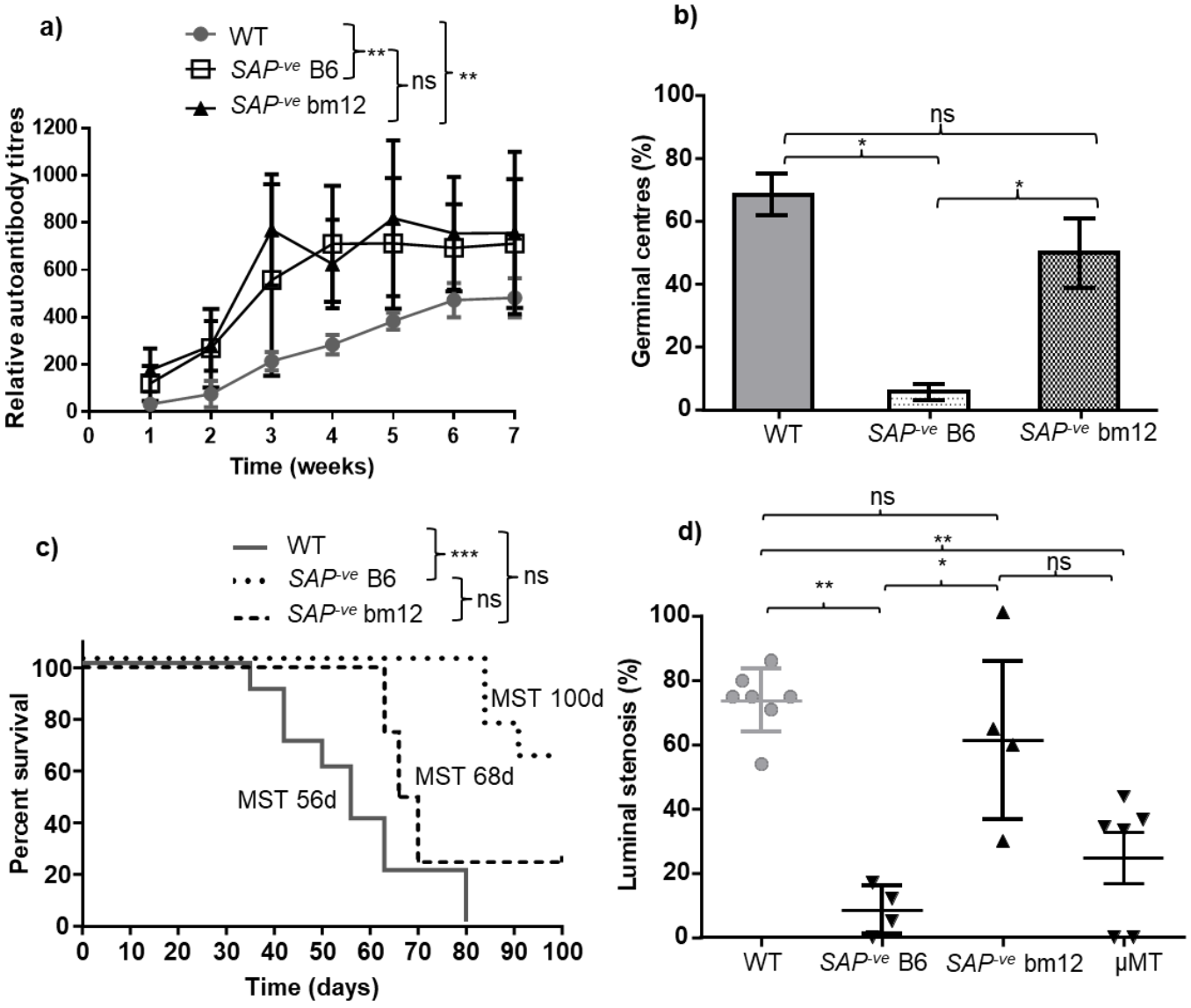

a)
WT
SAP-ve B6
SAP-ve bm12
**
ns
**
Relative autoantibody titres
Time (weeks)
b)
Germinal centres (%)
ns
*
*
WT
SAP-ve B6
SAP-ve bm12
c)
WT
SAP-ve B6
SAP-ve bm12
***
ns
ns
Percent survival
MST 100d
MST 68d
MST 56d
Time (days)
d)
ns
**
*
ns
**
Luminal stenosis (%)
WT
SAP-ve B6
SAP-ve bm12
µMT

Figure 5.5: Germinal centre autoantibody responses mediate progression of allograft vasculopathy

Wild-type (WT) or SAP-deficient (*Sh2d1a*$^{-/-}$) bm12 heart allografts were transplanted into wild-type (WT) or SAP-deficient (*Sh2d1a*$^{-/-}$) C57BL/6 (BL6) recipients (WT bm12 donor to WT BL6 recipient (WT); WT bm12 donor to *Sh2d1a*$^{-/-}$BL6 recipient (SAP^{-ve} BL6); *Sh2d1a*$^{-/-}$bm12 donor to WT BL6 recipient (SAP^{-ve} bm12).

a. Following transplantation, recipient anti-nuclear IgG autoantibody responses were measured weekly and expressed relative to positive hyperimmune control serum (1000 units) in three groups.

b. Frequency of splenic GCs, expressed as a proportion of total B cell follicles (histogram), in the various recipient groups. Primary follicles failed to differentiate into GCs when SAP signalling was inhibited in recipients (SAP^{-ve} BL6) but not when SAP signalling was inhibited in donors (SAP^{-ve}.bm12 group).

c. Kaplan-Meier curves depicting allograft survival.

d. At explant, the severity of allograft vasculopathy was determined morphometrically and expressed as percentage luminal stenosis (histogram), with representative photomicrographs of elastin van Gieson stained paraffin sections, depicting typical fibroproliferative arterial intimal thickening observed in rejecting heart allografts. Severe vasculopathy was only observed in groups in which germinal centre autoimmune responses were observed. Also the severity of AV in bm12 allografts was not different between SAP-ve BL6 recipients and B cell deficient (μMT) recipients.

Data represent mean and SD of n = 4-10 mice per group, with discrete data-points in **c** depicting individual animals. *P < 0.05, **P < 0.01 and ***P < 0.001 (2-way ANOVA in **a** Mann-Whitney test in **b** and **d**, and Kaplan-Meier log rank analysis in **c**).

5.4.4. Germinal centre autoantibody responses contribute to intermolecular epitope diversification

In order to investigate whether autoantibody is causing damage to the allograft, the allografts were examined for endothelial complement deposition by staining the frozen allograft sections with C4d staining. As reported previously (Win et al., 2009), the autoantibody responses generated in WT BL6 recipients of WT bm12 allografts were associated with C4d deposition in allografts (figure 5.6a). After confirmation of complement deposition in bm12 allografts following transplantation in WT BL6 recipients; complement deposition was investigated in all the other groups. Despite the development of antinuclear autoantibody and absence of a late GC response, complement deposition was not detected in bm12 allografts transplanted into SAP^{-ve} BL6 or TCR$^{bd-/-}$ recipients. However, this complement deposition was observed in allografts where GC autoantibody responses were seen; i.e. bm12 allografts transplanted into WT.BL6 CD4 T cell reconstituted TCR$^{bd-/-}$ recipients, and SAP^{-ve}.bm12 allografts into WT BL6 recipients (figure 5.6a).

This raised a question of whether germinal centres are contributing to maturation and diversification of autoimmune humoral response which in turn leads to progression of allograft vasculopathy. In order to examine this, in vitro assay was performed to test the ability of sera obtained from recipients from different groups at week 7 to induce migration/proliferation of cultured bm12 endothelial cells across a scratch wound in an attempt to evaluate the functional revelation of autoantibodies to induce EC migration and/proliferation because it may mimic migration of endothelial cells in response to antibody-mediated damage in vivo (Jin et al., 2014).

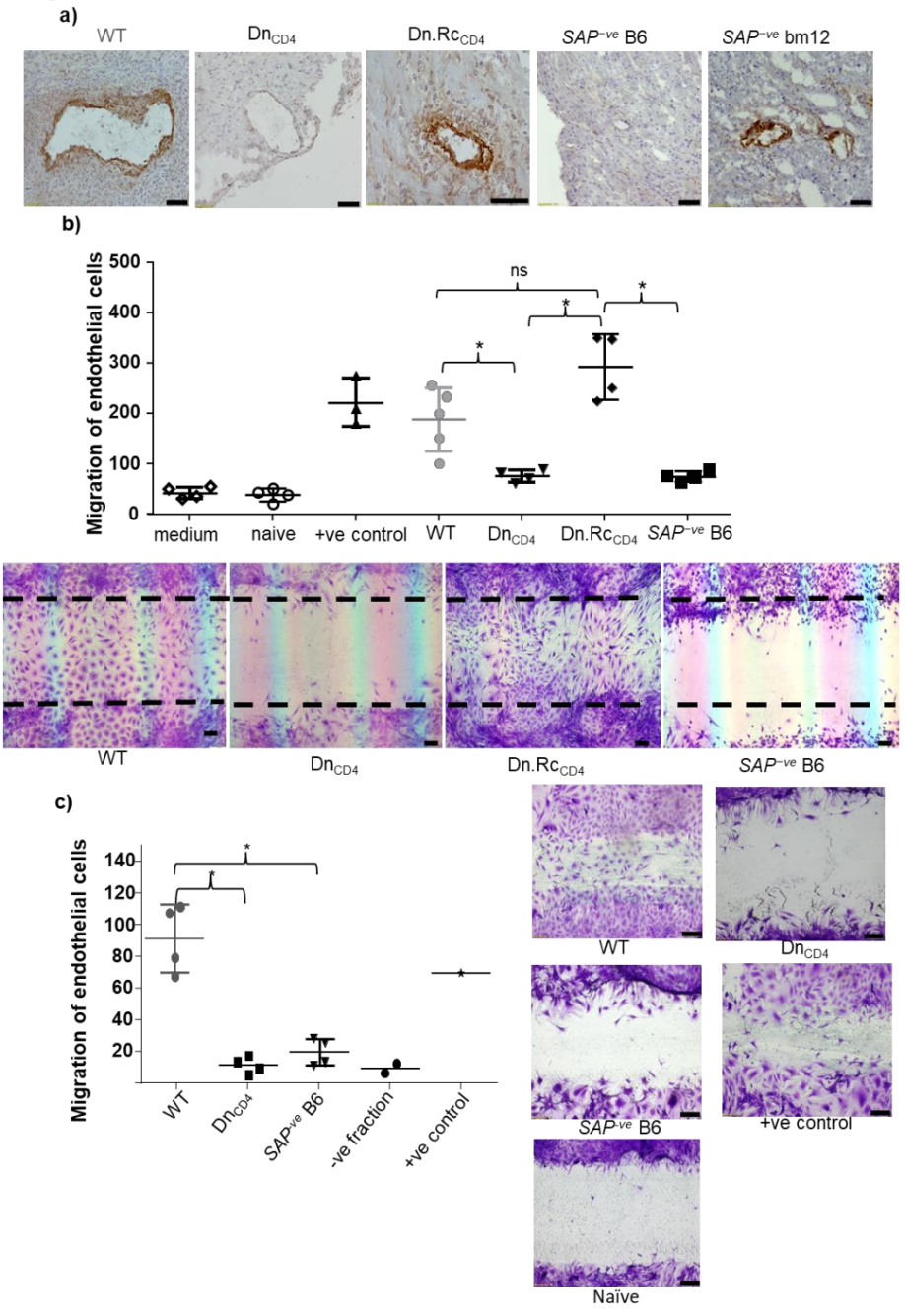
In order to perform in vitro endothelial migration assay, bm12 endothelial cells were cultured as described in methods (2.2.8) and a linear scratch was created in the centre then sera from different recipients were added and looked for migration/proliferation under low microscopy. Number of cells moved across the scratch was calculated. On analysis, sera from WT recipients of CD4 T cell-replete bm12 heart allografts triggered vigorous endothelial cell (EC) migration /proliferation, but in vitro EC responses were not observed with sera from either SAP^{-ve}.BL6 or TCR$^{bd-/-}$ recipients. However, sera from TCR$^{bd-/-}$ recipients reconstituted with WT.BL6 CD4 T cells restored the vigorous EC migration/proliferation; this was similar to that observed in WT recipients (figure 5.6b)(Qureshi et al., 2018). Furthermore, in order to remove any confounding factors to this assay because of the

presence of complement factors and other proteins in serum, autoantibody was column-purified (section 2.2.8.5) from recipients' sera from different groups and then used to assess the migration/proliferation of EC across a scratch wound. Not surprisingly, similar patterns of EC migration were observed when column-purified immunoglobulins from the transplanted recipients were added, with negative fraction from column purification not eliciting EC responses (figure 5.6c)(Qureshi et al., 2018). Thus GC autoantibody responses were able to trigger donor bm12 endothelial cells migration and/ or proliferation in vitro which may be mimicking in vivo graft damage by GC autoantibodies. From this we postulated that the requirement of GC autoimmunity in progression of allograft vasculopathy may be reflecting the diversification of humoral autoimmune responses that encompass and target additional and potentially pathogenic autoantigens over time after transplantation which may be detrimental to allografts.

To test diversification of humoral response, development of anti-vimentin autoantibody was checked in sera obtained from recipients from various groups at week7 and week 15.

As shown in chapter 3 (figure 3.10), there was a surge in anti-vimentin autoantibody at week 15 compared to week 7 in WT BL6 recipients of bm12 heart allografts. Interestingly, there was no detectable anti-vimentin autoantibody at week 7 in all the groups. However, there was a surge in anti-vimentin autoantibody response at week 15 in either WT recipients of SAP^{-ve}.bm12 heart grafts or $TCR^{bd-/-}$ recipients reconstituted with WT BL6 CD4 T cells. But this rise in anti-vimentin autoantibody at late time point was not observed in SAP^{-ve}.BL6 or $TCR^{bd-/-}$ recipients of bm12 heart grafts (figure 5.6d) (Qureshi et al., 2018).

Figure 5.6


a)
WT
Dn$_{CD4}$
Dn.Rc$_{CD4}$
SAP^{-ve} B6
SAP^{-ve} bm12
b)
Migration of endothelial cells
500
400
300
200
100
0
ns
*
*
*
medium
naive
+ve control
WT
Dn$_{CD4}$
Dn.Rc$_{CD4}$
SAP^{-ve} B6
WT
Dn$_{CD4}$
Dn.Rc$_{CD4}$
SAP^{-ve} B6
c)
Migration of endothelial cells
140
120
100
80
60
40
20
*
*
WT
Dn$_{CD4}$
SAP^{-ve} B6
-ve fraction
+ve control
WT
Dn$_{CD4}$
SAP^{-ve} B6
+ve control
Naïve

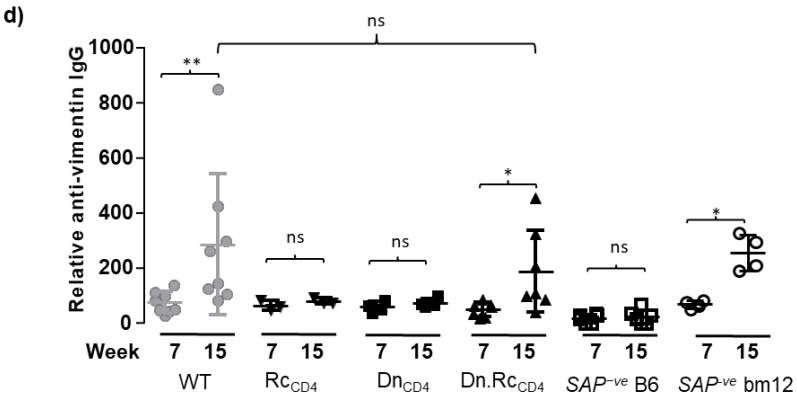

d)
Relative anti-vimentin IgG
1000
800
600
400
200
0
**
ns
ns
ns
*
ns
*
Week
7
15
WT
Rc$_{CD4}$
Dn$_{CD4}$
Dn.Rc$_{CD4}$
SAP^{-ve} B6
SAP^{-ve} bm12

Figure 5.6: Germinal centre autoantibody responses trigger endothelial cell responses and are associated with intermolecular epitope diversification

a. Immunohistochemical staining of explanted allografts reveals endothelial complement C4d deposition in donor recipient strain combinations in which late GC activity was observed (WT, Dn.RC$_{CD4}$, SAP^{-ve} bm12: group designations as in Figure 5.2 and 5.5), but not in strain combinations in which late GC activity was not present (Dn$_{CD4}$ and SAP^{-ve} BL6).

b. Endothelial C4d complement deposition correlated with the ability of week 7 sera from transplanted recipients to induce in vitro proliferation / migration of bm12 cultured endothelial cells in 'scratch-wound' assay (y-axis - number of cells encroaching into scratch). Representative photomicrographs of migration into the scratch wound are included. As positive control, commercial anti-H-2D^{b} mAb was added to endothelial cells.

c. In vitro proliferation / migration of bm12 cultured endothelial cells in 'scratch-wound' assay following addition of serum immunoglobulin (column-purified from sera of week 7 transplanted recipients). Migration was not observed with the negative fraction following column purification of sera from WT group (histogram). Representative photomicrographs of migration into the scratch wound are included. As positive control, commercial anti-H-2D^{b} mAb was added to endothelial cells.

d. Anti-vimentin IgG autoantibody responses in recipient groups were determined at weeks 7 and 15 after transplantation by indirect ELISA. Late anti-vimentin responses developed in those recipient groups in which GC activity was observed at week 7.

Data represent mean and SD of n = 4-8 mice per group, with discrete data-points in **b** & **c** depicting samples from individual animals. *P < 0.05, **P < 0.01 (Mann-Whitney test). Image scale bars are 50μm.

5.4.5. B cells are essential for diversification of humoral responses

In this model of chronic rejection, we have reported that donor CD4 T cells interact with MHC class II complexes on recipient B cells in a peptide degenerate fashion through direct allorecognition (Harper et al., 2016); however, it seemed logical that B cells would have to interact with recipient derived T_{FH} cells in a peptide-specific fashion in order to sustain autoreactive GCs as observed in WT recipients. To test this hypothesis, bone marrow chimeric mice were created with H2-DMa mice in $Rag2^{-/-}$ after sub-lethal irradiation as described in methods (2.1.9) (figure 5.7a). $Rag2^{-/-}$ does not have any B or T cells and H2-DMa mice have selective defect in B cells which were unable to exchange CLIP (class II-associated invariant chain peptide) and were impaired in their capacity to present endogenously processed peptides (Alfonso et al., 2001; Miyazaki et al., 1996). In the chimeric $Rag2^{-/-}$ mice, T and B cells would be from H-2DMa mice and DCs would be from H-2DMa and $Rag2^{-/-}$ mice, thus DCs would be providing an opportunity for initial priming of CD4 T cells and absence of DMa molecule on B cells would limit their ability to present a wider repertoire of endogenously processed peptides to CD4 T cells. After confirmation of chimerism in $Rag2^{-/-}$ showing presence of B cells, T cells and DCs (figure 5.7); H2-DMa chimeric mice were challenged with bm12 allografts. Recipients were checked for development of autoantibody response, GCs and anti-vimentin autoantibody(Qureshi et al., 2018).

On analysis of recipient sera; early autoantibody formed , which was above background levels in recipients of syngeneic BL6 grafts (figure 5.7b), confirming the peptide degenerate nature of the help provided by donor bm12 CD4 T cells to recipient B cells. In contrast to WT recipients, there was no augmentation of this autoantibody at late time point which was in consistent with failure of B cells to differentiate into GCs as shown by quantification of GCs at week 7 in recipients' splenic sections (figure 5.7c). This was in line with the fact that B cells were unable to present endogenous peptides; and peptide-specific help was unable to recruit T_{FH} cells for development of GC response. In the absence of GC response, diversification of B cells response to target anti-vimentin reactivity was not observed (5.7d) and allograft survival was prolonged (figure 5.7e) (Qureshi et al., 2018).

Figure 5.7

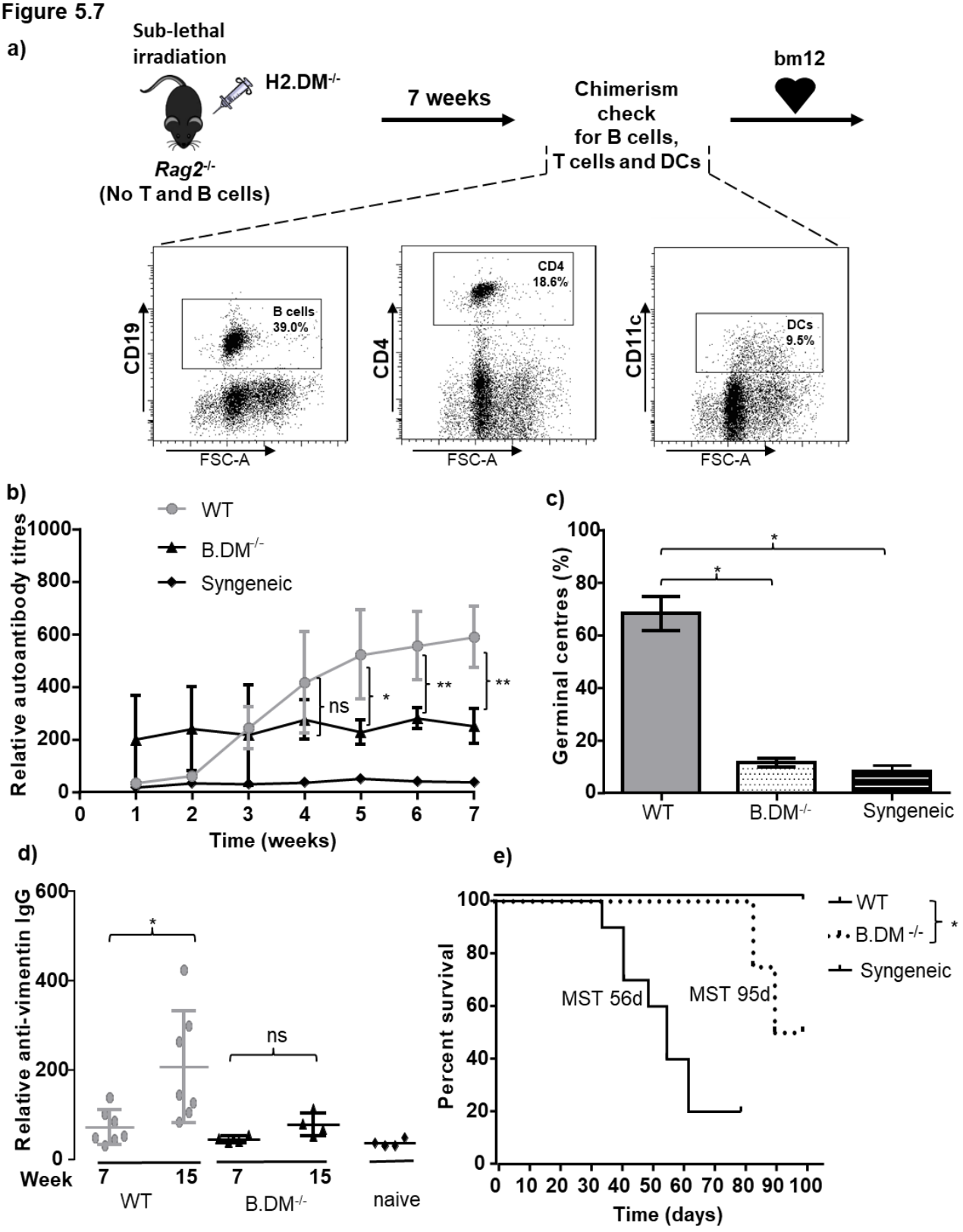

a)
Sub-lethal irradiation
H2.DM-/-
Rag2-/-
(No T and B cells)
7 weeks
Chimerism check for B cells, T cells and DCs
bm12
CD19
B cells 39.0%
FSC-A
CD4
CD4 18.6%
FSC-A
CD11c
DCs 9.5%
FSC-A
b)
WT
B.DM-/-
Syngeneic
Relative autoantibody titres
Time (weeks)
ns
*
**
**
c)
Germinal centres (%)
WT
B.DM-/-
Syngeneic
d)
Relative anti-vimentin IgG
Week
7
15
WT
B.DM-/-
naive
ns
e)
Percent survival
MST 56d
MST 95d
WT
B.DM -/-
Syngeneic
Time (days)

Figure 5.7: B cell antigen presentation is required for germinal centre autoimmunity

- **a.** H-2^b mice with an isolated defect in B cell antigen presentation (B.$DM^{-/-}$) were created by adoptive transfer of bone marrow from *H-2DM* genetically-deficient mice into sub-lethally irradiated *Rag2*$^{-/-}$ mice. Flow cytometry plots, gating on live lymphocyte fraction (left and middle) and macrophage / dendritic cell fraction (right), demonstrating that the resultant B-$DM^{-/-}$ mice have restored expression of $CD19^{+ve}$ B cells and $CD4^{+ve}$CD4 T cells (gated numbers represent percentage of live lymphocytes). Whereas all B cells will be defective for H-2DM expression, other antigen presenting cell subsets will partially originate from the H-$2DM^{+/+}$ *Rag2*$^{-/-}$ bone marrow fraction and hence are capable of relatively normal antigen presentation.
- **b.** Following transplantation of B-$DM^{-/-}$ mice with bm12 heart allografts, antinuclear autoantibody responses
- **c.** germinal centre activity
- **d.** anti-vimentin autoantibody responses
- **e.** and allograft survival

All the above were assessed as described for Figures 5.4 and 5.5 and compared to responses observed in wild-type (WT) BL6 and recipients of syngeneic (BL6) transplants.

Data represent mean and SD of n = 4-10 mice per group, with discrete data-points in **d** depicting samples from individual animals. $^{*}P < 0.05$, $^{**}P < 0.01$ (2-tailed t-test for individual time-points in **b**, Mann-Whitney test in **c** and **d,** and Kaplan-Meier log rank analysis in **e**).

5.4.6. Late B cell depletion prevents epitope diversification and prolongs allograft survival

To further address the role of GC in diversification; we hypothesised that once GCs have developed, inhibition of GC B cells to mature and mutate to form diversified humoral response would curtail the diversification phenomenon. To test this, WT BL6 recipients of bm12 allografts were treated with depleting anti-CD20 monoclonal antibody, on days 25, 35 and 45 after transplant, with depletion confirmed by flow cytometric analysis of peripheral blood (Ali et al., 2016). Anti CD-20 monoclonal antibody would deplete immature, mature established GC B cells but would be ineffective against autoreactive plasma cells, such as those that are resident in bone marrow as a result of initiation of germinal centre response (Hoyer et al., 2005). B cell depletion was initiated at week three due to the fact that in this model GC develop around day 15 (chapter 3, figure 3.7) and we wanted to block GC B cells once they have developed, thus preventing diversification of humoral response, but we can still see GC output in the form of bone marrow plasma cells. In support of this, late treatment with anti-CD20 did not affect deposition of long lived plasma cells specific for double-stranded DNA (dsDNA) into the bone marrow which is consistent with the comparable autoantibody titres between treated and untreated recipients (Figure 5.8a). Splenic GC activity was however less than the WT (figure 5.8c) and it was consistent with abrogation of late anti-vimentin response (figure 5.8d). All donor allografts survived till day 100 with minimal vasculopathy (figure 5.8e). However, donor hearts were beating appreciably less strongly prior to explant. Histologic assessment revealed marked cellular infiltrate within allografts and was associated with fibrotic scarring (figure 5.8f) (Qureshi et al., 2018).

Figure 5.8

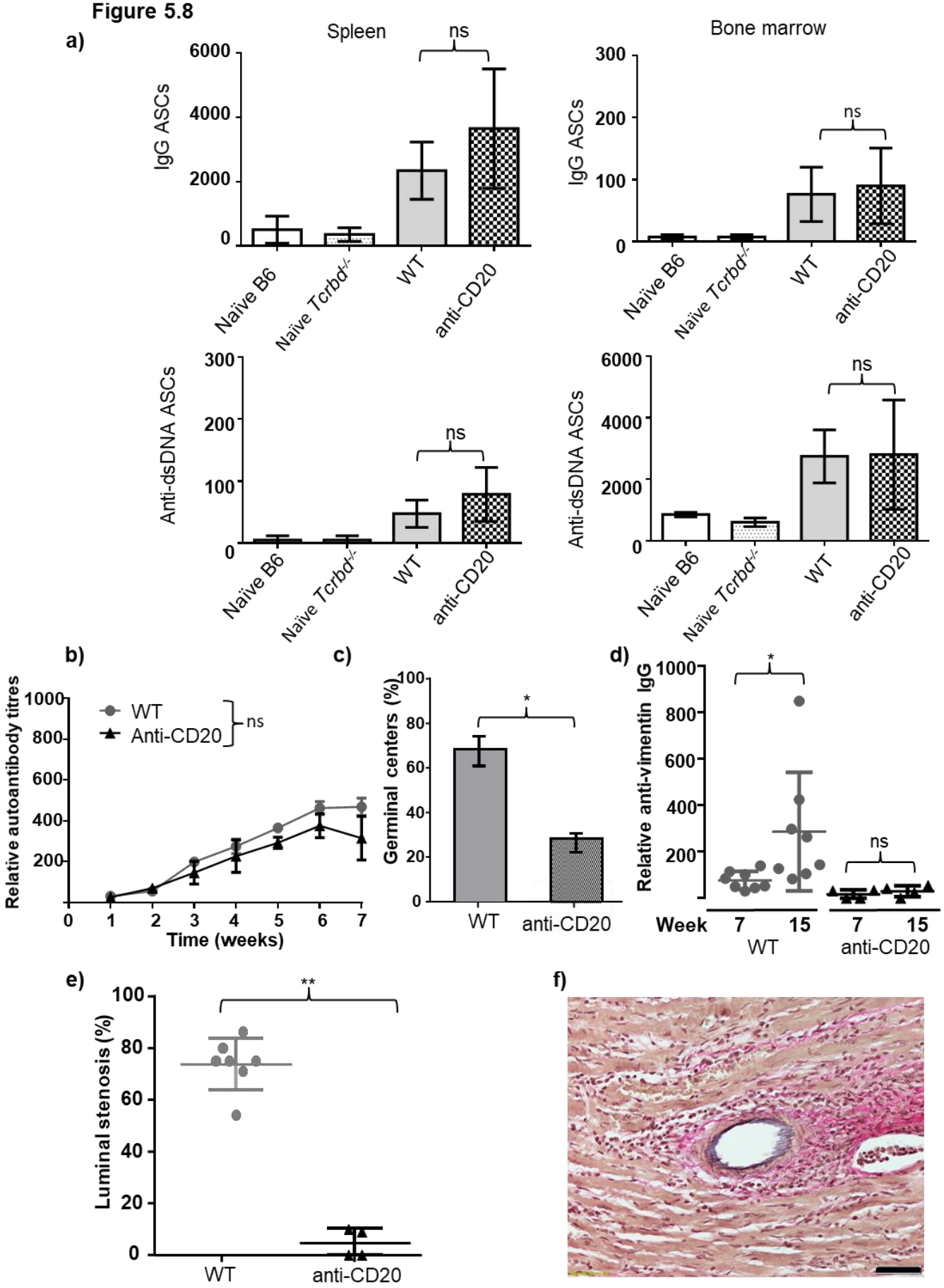

a)
Spleen
Bone marrow
IgG ASCs
Anti-dsDNA ASCs
ns
Naïve B6
Naïve Tcrbd-/-
WT
anti-CD20
b)
Relative autoantibody titres
WT
Anti-CD20
Time (weeks)
c)
Germinal centers (%)
d)
Relative anti-vimentin IgG
Week
e)
Luminal stenosis (%)
f)

Figure 5.8: Late B cell depletion prevents epitope diversification and improves allograft survival

C57BL/6 (BL6) recipients were treated with depleting anti-CD20 on days 25, 35 and 45 after transplantation with bm12 heart allografts.

a. Seven weeks after transplantation, IgG-secreting (top row) and anti-double-stranded DNA (dsDNA, bottom row) antibody secreting cells were enumerated in spleen (left) and bone marrow (right) by standard ELISPOT assay and expressed as antibody secreting cells (ASCs)/ million cells plated. Shown for comparison are responses in wild-type BL6 recipients of wild-type bm12 heart allografts (wild-type) and those observed in naïve, untreated wild-type and *Tcrbd*$^{-/-}$ BL6 mice **(a)**.
b. Anti-nuclear IgG autoantibody responses in anti-CD20 treated recipients were similar to those observed in untreated recipients
c. Frequency of splenic GCs seven weeks after transplant was reduced in anti-CD20 treated groups compared to WT.
d. **(c)**. The reduction in GC was associated with amelioration of the late anti-vimentin autoantibody response in treated group (anti-CD20) vs untreated group (WT)
e. Donor grafts were assessed at explant at week 7 for AV which revealed significant reduction in AV in treated group.
f. Elastin van Gieson stained paraffin sections of allografts at explant revealed widespread foci of cellular infiltration (**f**, scale bars 50μm).

Data represent mean and SD of n = 4-8 mice per group, with discrete data-points in **d & e** depicting samples from individual animals. **P* < 0.05 (2-way ANOVA in **b**, and Mann-Whitney test in **a**, **c**, **d** & **e**).

5.4.7. Germinal centre autoantibody responses independently mediate progression of allograft vasculopathy

So far, I have shown the definite role of recipient CD4 T cells, acting as a T_{FH} cells for maintaining and propagating donor induced autoimmunity through germinal centre formation. This GC autoimmunity in turn is contributing to progression of allograft vasculopathy especially in the light of our previous work in which we have shown that BL6 recipients of bm12 heart allografts do not develop alloantibody responses against mismatched I-A^{bm12} MHC class II antigen (Win et al., 2009). However, we want to exclude any possible contribution to allograft rejection from conventional recipient CD4 T cell allorecognition of the mismatched I-A^{bm12} alloantigen either through direct or indirect pathway (Ali et al., 2013). In order to examine this, we created 'mixed chimeric mice' modified from Sykes' work (Nikolic et al., 2010). This mixed chimeric mice was created by adoptive transfer of bone marrow from CD45.2.bm12 mice into CD45.1.BL6 with CD4 and CD8 depleting antibodies and anti-CD154 antibodies at various time points (method section 2.1.8.1). The recipient mice were tail bled at week 7 to perform flow cytometry to check the chimeric state which confirmed that CD45.1.BL6 mice stably embraced a congenic population of CD45.2.bm12 bone marrow (Chm.bm12/BL6 mice) (figure 5.9a). We hypothesised that in these mice the persistence of stable chimeric state would signify ongoing adaptive tolerance towards the mismatched MHC-class II alloantigens, however a subsequent challenge with bm12 allograft to the recipients will break this tolerance and by breaking the tolerance donor CD4 T cells in the allografts will provide help to recipient B cells and result in autoimmunity.

Mixed chimeric mice were challenged with bm12 allograft and recipients' sera were checked for antinuclear autoantibody and late anti-vimentin autoantibody levels, recipient splenic sections were looked for germinal centres and donor allograft vasculopathy were calculated at explant.

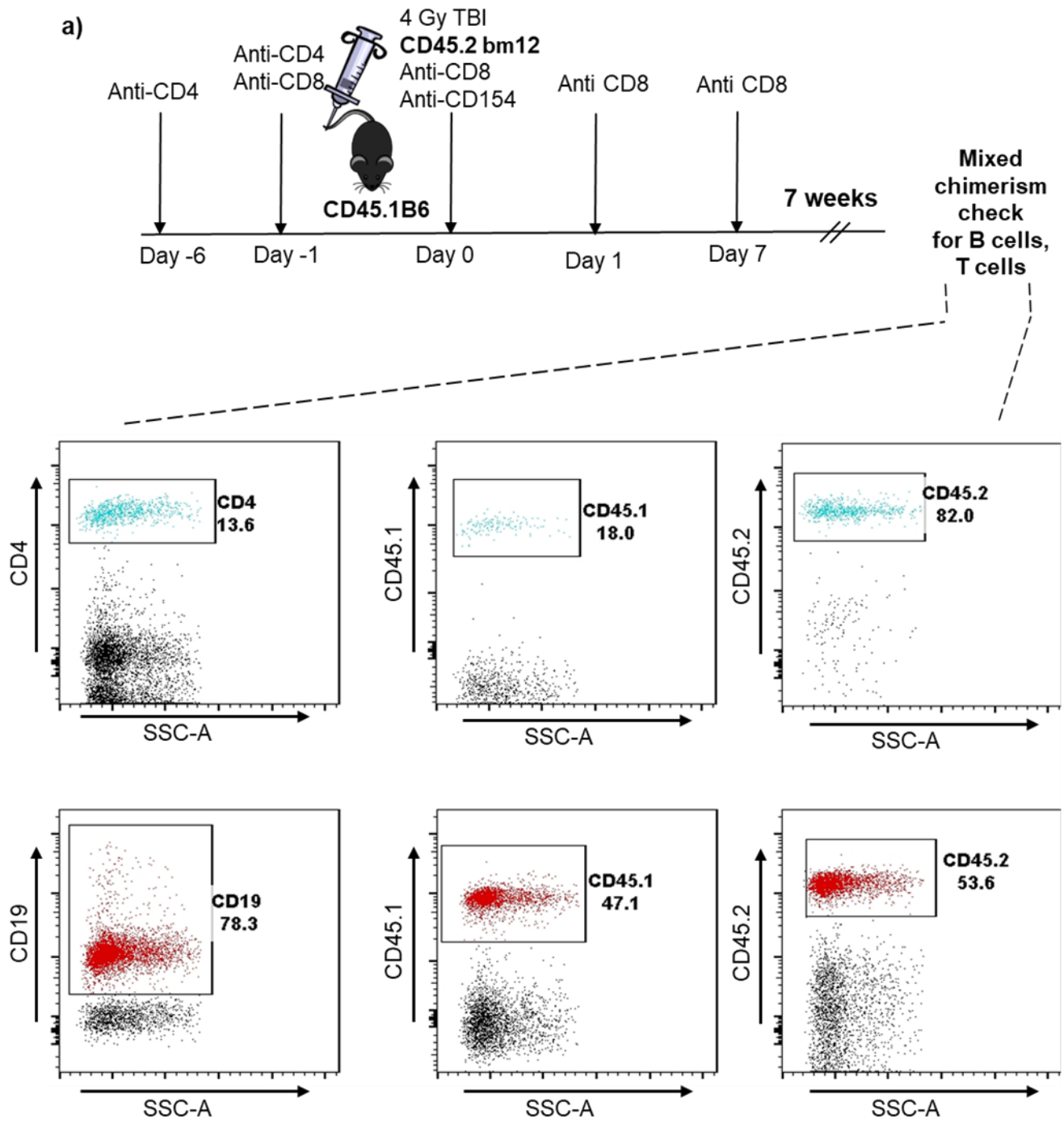
Antinuclear autoantibody response was robust (figure 5.9b) and long-lasting comparable to WT responses; this was in keeping with germinal centre response (figure 5.9c). Allografts developed severe vasculopathy (figure 5.9e) and rejected at the same tempo as that of WT (figure 5.9f). Allografts also demonstrated wide spread complement deposition (figure 5.9g).

Analysis of vimentin revealed that late anti-vimentin autoantibody was above background level (figure 5.9d).

The above results suggested that donor CD 4 T cells were capable of breaking B cell tolerance despite stable tolerant state to the mismatched I-Abm12 alloantigen that was present at the transplant. Passenger CD4 T cells within the allograft stimulated GC mediated humoral autoimmune response which resulted in progressive allograft vasculopathy and eventual allograft failure. However, it did not exclude the possible role of autoimmunity in breaking the allotolerance in the chimeric mice or possible contribution of recipient adaptive alloimmune responses generated against the MHC class II I-A^{bm12} alloantigens towards chronic allograft failure.

Furthermore, as we have reported previously (Harper et al., 2016) that despite the development of a robust GC autoimmunity response in the recipients in this model of chronic rejection (bm12 to BL6); the recipients did not develop any obvious signs of chronic graft versus host disease within the time frame of our experiments. This raised a question why the autoantibody formed in recipients is damaging the donor arterial system and thus causing allograft vasculopathy but apparently preserving the native arterial system. Interestingly, when syngeneic BL6 heart grafts were transplanted into BL6 recipients in which humoral autoimmunity was simultaneously provoked by adoptive transfer of purified bm12 CD4 T cells at the day of transplant, the heart grafts survived indefinitely without developing any vasculopathy (figure 5.9e). These experiments suggested that ischaemic-reperfusion injury was not itself sufficient to induce susceptibility in the syngeneic transplant to autoantibody mediated damage (Qureshi et al., 2018).

Figure 5.9


a)
Anti-CD4
Anti-CD4
Anti-CD8
4 Gy TBI
CD45.2 bm12
Anti-CD8
Anti-CD154
Anti CD8
Anti CD8
CD45.1B6
7 weeks
Day -6
Day -1
Day 0
Day 1
Day 7
Mixed chimerism check for B cells, T cells
CD4
13.6
CD4
SSC-A
CD45.1
18.0
CD45.1
SSC-A
CD45.2
82.0
CD45.2
SSC-A
CD19
78.3
CD19
SSC-A
CD45.1
47.1
CD45.1
SSC-A
CD45.2
53.6
CD45.2
SSC-A

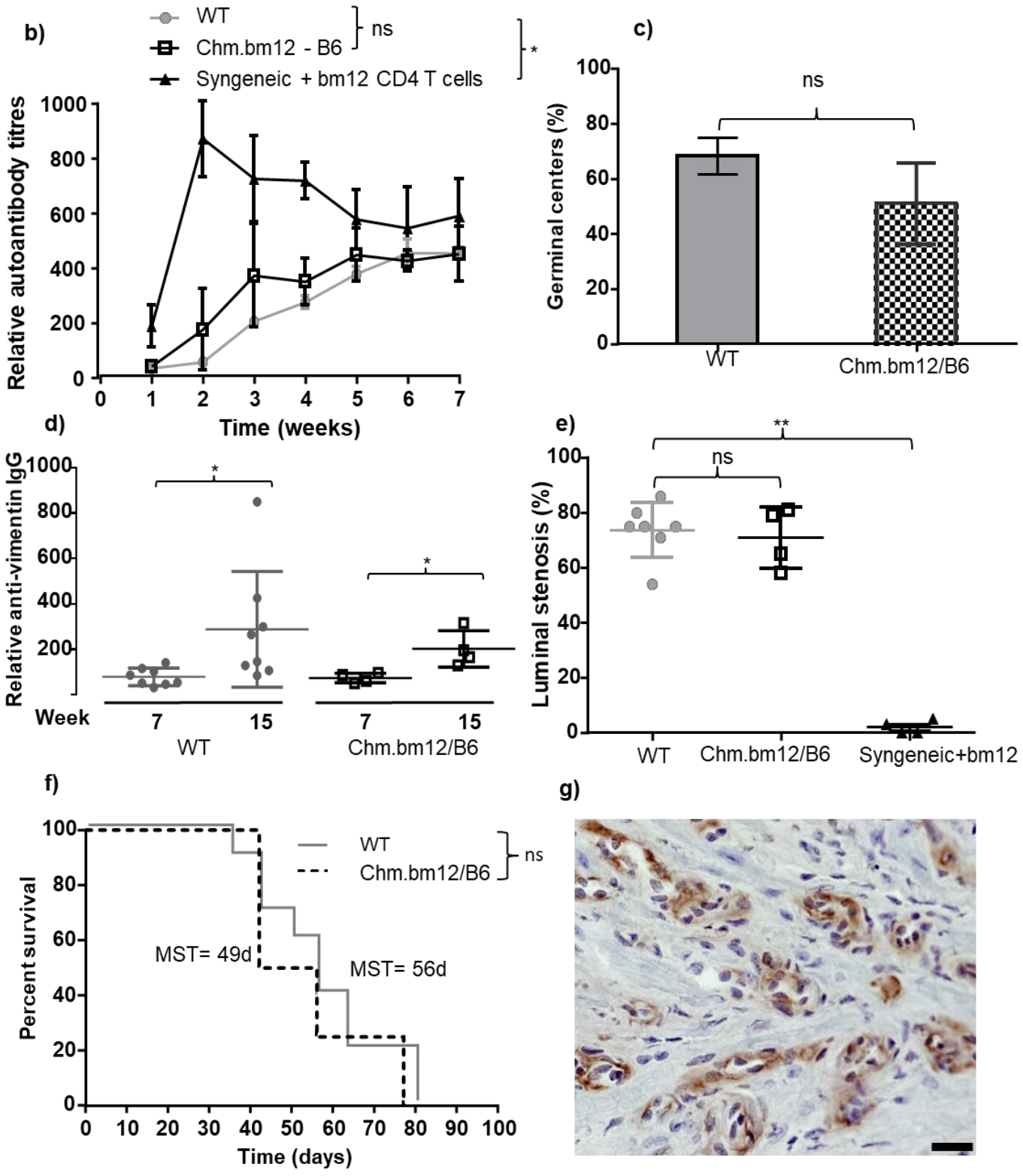

b)
WT
Chm.bm12 - B6
Syngeneic + bm12 CD4 T cells
ns
*
Relative autoantibody titres
Time (weeks)
c)
Germinal centers (%)
ns
WT
Chm.bm12/B6
d)
Relative anti-vimentin IgG
*
*
Week
7
15
7
15
WT
Chm.bm12/B6
e)
**
ns
Luminal stenosis (%)
WT
Chm.bm12/B6
Syngeneic+bm12
f)
Percent survival
WT
Chm.bm12/B6
ns
MST= 49d
MST= 56d
Time (days)
g)

Figure 5.9. Donor: recipient bone-marrow chimeric mice reject donor strain heart allografts

- **a.** Creation of mixed haematopoietic chimeric mice**,** stable bm12→C57Bl/6 mixed haematopoietic bone marrow chimeric mice were created by conditioning sub-lethally irradiated (4 Gy total body irradiation) congenic CD45.1 BL6 mice with anti-CD8, anti-CD4, and anti-CD154 mAbs, followed by intravenous injection with CD45.2 bm12 bone marrow cells (diagram). The presence of mixed haematopoietic chimerism was assessed 7 weeks later by flow cytometric analysis of peripheral blood mononuclear cells (representative histogram plots depicted), and identifying bm12 donor (CD45.2) and BL6 recipient (CD45.1) fractions within gated T cell (top row) and B cell (bottom row) populations. Mixed stable chimeric recipients were challenged with bm12 donor allo-grafts and the results were compared to those achieved in wild-type C57BL/6 recipients of a bm12 heart allograft (WT.BL6) and to C57BL/6 recipients transplanted with a syngeneic C57BL/6 heart allografts and challenged intravenously with purified bm12 CD4 T cells and (syngeneic+bm12).
- **b.** Antinuclear autoantibody levels, which were comparable to WT and syngeneic+bm12
- **c.** Frequency of splenic germinal centre activity at week 7, comparable to WT.
- **d.** Late anti-vimentin IgG autoantibody response was present in chimeric recipients.
- **e.** Allograft vasculopathy of week 7 explants, the severity of AV of allografts transplanted in chimeric recipients was the same as that of WT but no donor AV in syngeneic+bm12 group.
- **f.** Allografts were rejected at the same tempo as that of WT.
  b, c, d, e and f were assessed as described in Figures (5.2, 5.4 and 5.5) and
- **g.** At explant at 7 weeks, cryostat immunohistochemistry of donor grafts confirmed widespread endothelial C4d complement deposition in chimeric recipients; scale bar 20μm.

Data represent mean and SD of n = 4-10 mice per group, with discrete data-points in **c & d** depicting samples from individual animals**.** **P* < 0.05 (2-way ANOVA in **a**, Mann-Whitney test in **b**, **c** and **d**, and Kaplan-Meier log rank analysis in **e**).

To definitely examine the role of humoral autoimmunity independent of alloimmune response in development of allograft vasculopathy, we selected to investigate the rejection of parental strain heart allograft by F1 hybrid offspring (Uehara et al., 2005). Previously (Uehara et al., 2005), it has been suggested that NK cells contribute to rejection of parental allografts when transplanted into F1 hybrid offspring by generation of IFN-γ from recipients. In this transplant model, anti-donor T cell reactivity lacks but it retains anti-donor NK cell responses. They further suggested that amelioration of AV in donor heart grafts by inhibition of recipient NK cells by treatment of recipients by anti-NK 1.1 monoclonal antibody argues that NK cells activated by the absence of self MHC class I molecules on donor endothelium participate in the pathogenesis of AV. However, autoimmune responses were not studied in that study. Hence, we hypothesised that in this model graft versus host allorecognition by passenger donor cells would trigger recipient GC autoimmunity, but that adaptive alloimmune recognition of donor parental antigens would not be possible in the F1 recipients.

In order to do that, transplantation of BALB/c heart grafts with or without CD4 T cells into F1 BALB/c x C57BL/6 (CB6F1) recipients were carried out. Recipients' sera was checked for development of antinuclear autoantibody responses, recipient splenic sections were examined for development of GCs, donor heart allografts were calculated for development of allograft vasculopathy at explant and also examined for complement deposition. Transplantation of BALB/c grafts into CB6F1 resulted in development of long lasting autoantibodies in the recipients (figure 5.10a), which was in keeping with the presence of germinal centres in recipient splenic sections (figure 5.10c). These GC autoimmune responses were associated with development of allograft vasculopathy (figure 5.10d) and intra-allograft endothelial complement deposition (figure 5.10e). However, alloantibody responses against MHC class I H-2K^{d} antigen were not generated, suggesting that self-tolerance to the BALB/c H-2^{d} antigens was maintained in the F1 recipients (figure 5.10b). As expected, these GC autoimmune responses were absent when CD4 T cells depleted BALB/c donors were transplanted into CB6F1 recipients (figure 5.10a and c) and explanted donor allografts showed minimal allograft vasculopathy (figure 5.10d) without complement deposition (figure 5.10e). These findings were consistent with the requirement of donor CD4 T cells to stimulate GC mediated autoimmunity in recipients. In order to further evaluate the

interplay between donor and recipient CD4 T cells for development of this donor induced autoimmunity in the recipients, *Sh2d1a*$^{-/-}$*.F1 (*SAP^{-ve} F1) recipients were created as mentioned in methods (2.1.1) with the expectation that inhibition of SAP signalling in the recipients will block the recipient CD4 T cells to provide help to the activated B cells to form germinal centres and thus will ameliorate the AV in donor heart grafts as was seen in bm12 to BL6 model.

Interestingly, transplantation of BALB/c donor allograft into *Sh2d1a*$^{-/-}$*.F1 (*SAP^{-ve} F1) recipients abrogated the GC response (figure 5.10a and c) and heart grafts developed minimal vasculopathy (figure 5.10d) with no complement deposition in intra-allograft endothelial cells (figure 5.10e).

Then we investigated the effect of NK cells on rejection kinetics in this model by depleting NK cell in the recipients. We presumed that depleting NK cells in the recipients would augment humoral autoimmune response. Depletion of recipient NK cells was carried out as described previously (Ali et al., 2016) and in methods (2.1.6). Transplantation of BALB/c allografts to NK-cell depleted recipients although resulted in GC autoantibody responses but the allografts remained disease free without any complement deposition. This suggested a critical role for NK cells in either mediating alloantibody or autoantibody mediated graft damage or in triggering innate immune 'missing self' recognition of the parental BALB/c strain.

Figure 5.10

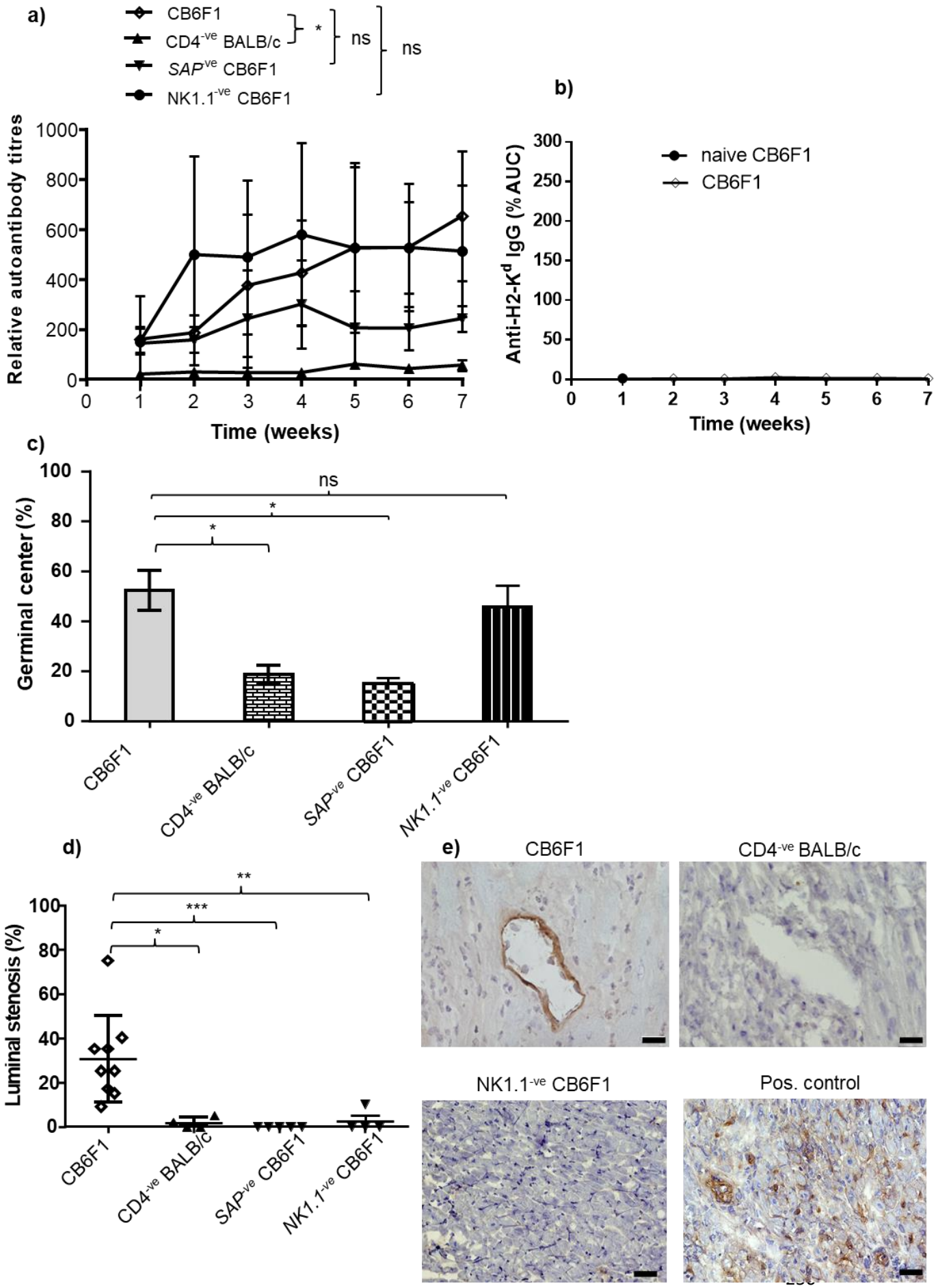

a)
CB6F1
CD4-ve BALB/c
SAPve CB6F1
NK1.1-ve CB6F1
*
ns
ns
Relative autoantibody titres
Time (weeks)
b)
naive CB6F1
CB6F1
Anti-H2-Kd IgG (% AUC)
Time (weeks)
c)
Germinal center (%)
ns
*
*
CB6F1
CD4-ve BALB/c
SAPve CB6F1
NK1.1-ve CB6F1
d)
Luminal stenosis (%)
**
***
*
CB6F1
CD4-ve BALB/c
SAPve CB6F1
NK1.1-ve CB6F1
e)
CB6F1
CD4-ve BALB/c
NK1.1-ve CB6F1
Pos. control

Figure 5.10: Germinal centres autoantibody responses independently mediate progression of allograft vasculopathy.

Wild-type BALB/c heart allografts were transplanted into either unmodified BALB/c x C57BL/6 F1 (CB6F1) mice or CB6F1 mice genetically deficient in expression of SAP (SLAM associate protein (SAP^{-ve} CB6F1)) or CB6F1 mice depleted of NK T cells ($NK1.1^{-ve}$ CB6F1). Heart grafts from CD4 T cell-depleted BALB/c donors were transplanted into unmodified CB6F1 recipients ($CD4^{-ve}$ BALB/c).

a. Following transplantation, anti-nuclear IgG autoantibody by hep-2 indirect immunofluorescence on recipient sera.
b. Following transplantation, anti-Kd alloantibody in recipient splenic sera by H-2 Kd ELISA.
c. Recipient splenic GC activity till week 7.
d. Donor allograft vasculopathy at week 7.

a), c), d) were assessed as described in Figures 1 & 2.

e. Cryostat immunohistochemistry of heart allografts at week 7 revealed widespread endothelial C4d complement deposition in CB6F1 recipients, but not in $CD4^{-ve}$ BALB/c allografts nor in $NK1.1^{-ve}$ CB6F1 recipients. Scale bar 20µm.

Data represent mean and SD of n = 4-9 mice per group, with discrete data-points in **d** depicting samples from individual animals**.** *$P < 0.05$ (2-way ANOVA in **a**, and Mann-Whitney test in **c** & **d**).

5.5. Summary

In summary, this work makes several important and novel observations (figure 5.11)

- Recipient humoral autoimmunity is triggered by graft-versus-host recognition by donor CD4 T cells, but its maintenance as a germinal centre response requires help from CD4 T cells in the recipient that have undergone follicular helper T cell differentiation.
- Humoral autoimmunity can contribute to allograft rejection, but only as a germinal centre response. Germinal centre response is associated with epitope diversification to target additional autoantigens.
- Germinal centre autoantibody responses can effect allograft rejection independently of host alloimmunity.

Figure 5.11

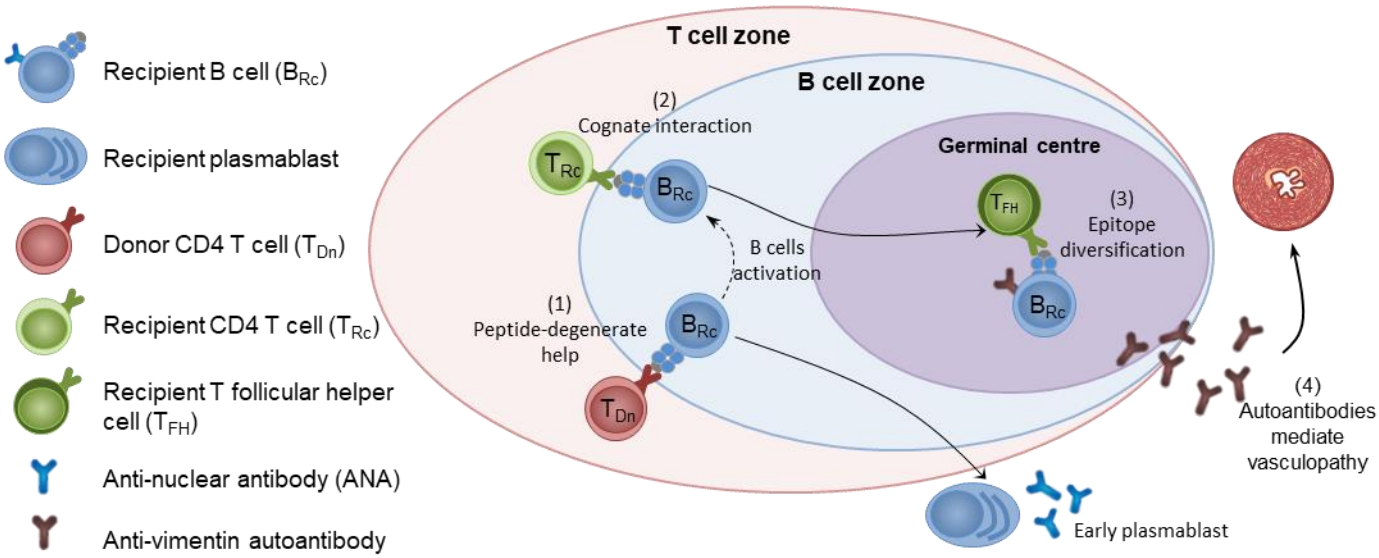


Figure 5.11: **Proposed model for interaction between donor CD4 T cells and recipient B and T cells for initiation and maintenance of germinal center autoimmunity**

Passenger donor CD4 T cells within the heart allograft provide 'peptide-degenerate' help to host B cells for production of anti-nuclear IgG autoantibody (1). Subsequent 'cognate' interaction between activated B cells and recipient T follicular helper (T_{FH}) cells results in long-lasting germinal center responses (2), which lead to inter-molecular epitope diversification, to encompass, along with other targets, responses against vimentin autoantigen (3). This diversification is associated with development of antibody-mediated allograft vasculopathy and eventual graft failure (4).

5.6. Discussion

Although the development of autoimmunity has been increasingly associated with chronic rejection of organs especially following heart and lung transplantation; how this autoimmunity contributes to allograft rejection remains poorly understood. Previous studies from us and other groups have shown that graft specific autoimmunity affects allografts in close concert with adaptive alloimmune responses which are directed against mismatched MHC alloantigens. This contribution from autoimmune response is described as either precipitating or augmenting alloimmune response towards graft damage (Cardinal et al., 2013; Harper et al., 2016; Lovegrove et al., 2001; Yoshida et al., 2006) or this autoimmune response itself may be the result of ongoing vigorous alloimmune mediated graft damage (Fukami et al., 2009; Valujskikh et al., 2002). In this chapter I have extended our previous investigations into bm12 to BL6 model of heart allograft vasculopathy, and have shown that although host autoantibody responses were stimulated by donor CD 4 T cells, this donor CD4 T cells induced autoimmunity in the recipient was maintained by provision of help to recipient B cells from recipient derived T_{FH} cells which helped recipient B cells to differentiate into germinal centres. Maintenance and propagation of this donor induced autoimmunity by recipients' T_{FH} cells through GC autoantibody response is, in turn, a major determinant of graft outcome (figure 5.5c and d). Furthermore, by replicating these findings with another model in which the host adaptive alloimmune response against the donor was not possible (figure 5.10), I have provided a strong evidence for an independent causative role for graft specific autoimmunity in chronic organ rejection.

Bm12 to BL6 model has been one of the most studied models of chronic rejection due to minimal mismatch between donor and recipients. However, surprisingly, role of autoantibody has not been described. Nevertheless, our previous studies (Harper et al., 2016; Motallebzadeh et al., 2012; Win et al., 2009) and the current data continue to emphasize the contribution of autoimmune humoral response to graft damage. The most salient feature of this work is the presence of robust GC responses in BL6 recipients splenic sections of bm2 allografts at week 7 and the absence of this GC autoimmune response in SAP^{-ve} BL6 recipients of bm12 allografts. This failure of recipient B cells to differentiate into GC autoimmune responses in SAP^{-ve} recipients resulted in reduction in AV and prolongation

of bm12 allograft survival. SAP^{-ve} mice have normal B and T cell function apart from some defect in NK T cells and T_{FH} cells (Czar et al., 2001). Hence in this model, in which there is no demonstrable alloantibody against donor I-A^{bm12} (Win et al., 2009) and short lived direct pathway response against the disparate I-A^{bm12} MHC class II (Ali et al., 2016), it seems likely that robust and long lasting GC autoantibody response which is dependent on recipient T_{FH} cells is contributing to allograft chronic rejection. However, autoimmunity does not seems to be the only mediator of AV as AV does develop in B cell deficient recipients (figure 5.5d) (Win et al., 2009) but with less severity. Furthermore, from this work, an additional role of cellular alloimmunity in graft rejection has been suggested from late B cell depleted recipients work in which bm12 allografts demonstrated minimal AV but presence of cellular infiltrates in parenchyma with fibrosis and a decrease in heart beating noticed at the time of explant. This piece of work suggests that humoral autoimmunity mediates progression of graft damage by affecting graft endothelium and cellular alloimmunity effect graft parenchyma (Fischbein et al., 2001).

The demonstration that donor CD4 T cells stimulate GC autoimmunity in the recipients and recipient CD4 T cells are acting as a T_{FH} cell for durable GCs which is the major determinant for progression of allograft vasculopathy, raised several questions which are unanswered; why is the donor population of CD4 T cells unable to provide T_{FH} cell function to support long term GCs; what is the nature of antigen recognition by recipient CD4 T cells and how does the GC autoimmune response contribute to allograft rejection.

With regards factor which may be preventing donor bm12 CD4 T cells to acquire T_{FH} cell function we have had a detailed discussion in previous chapter with adoptive transfer of CD4 T cells. Importantly two of the hypotheses in this regard have been tested as well (figure 4.23 and 4.24). Firstly, my work has suggested that donor bm12 CD4 T cells were unable to provide long term T_{FH} cell function due to their direct interaction with recipient B cells through their MHC class II in a peptide degenerate fashion which is non-specific and low affinity interaction (Sewell, 2012) (figure 4.23). Secondly, again this direct allorecognition of MHC II on DCs by donor bm12 CD4 T cells may result in downregulation of Bcl-6 which may limit their ability to provide long term T_{FH} cell function (figure 4.24). Furthermore, we discussed that it may be possible that long term GC maintenance may

require renewal of the existing T_{FH} cells by continual differential of CD4 T cells into new T_{FH} cells to the already existing GC (figure 4.22) (Shulman et al., 2013).

The differentiation of recipient CD4 T cells into T_{FH} cell is probably driven by antigen presentation from recipient activated B cells following their first interaction with donor bm12 CD4 T cells. As B cells are capable of soliciting their own help (Stockinger et al., 1996) and it is quite possible that once B cells are activated by donor CD4 T cells then they would be presenting self-restricted peptide autoantigens on MHC class II to recipient CD4 T cells and by doing so would be recruiting naïve autoreactive recipient CD4 T cells for differentiation into T_{FH} cells and ultimately for graft rejection. This provides an explanation why in the bm12 to BL6 model, a monoclonal population of host direct pathway CD4 T cells was found to be unable to mediate allograft rejection (Sayegh et al., 2003).

The observed complement deposition in allograft and in vitro migration of donor endothelial cells to autoantibody reflects that the damage caused by GCs is primarily dependent on the quality of autoantibody produced as there was no migration in groups where B cells failed to differentiate into GCs. This reflects GC B cells ability to produce mutated plasma cells through somatic hypermutation which in turn secret high affinity autoantibodies coupled with deposition of mutated plasma cells into bone marrow as a continual supply of high affinity autoantibody for life time. In support, dsDNA specific bone marrow plasma cells were associated with GC response in WT recipients of bm12 donors (figure 5.8a). Presence of autoimmune responses to vimentin autoantibody at a late point suggests the possibility of epitope diversification to encompass additional pathogenic autoantigens, may also be an important function of GC in mediating graft rejection. However, my work does not conclusively prove this diversification phenomenon from other attributes of GCs. Furthermore, in our experiments it does not quite show that it is specifically involved in graft damage as it developed after graft rejection and half of the recipients did not develop this autoantibody. However, we selected this as a surrogate marker of diversification due to previous studies which have shown its contribution to allograft vasculopathy (Mahesh et al., 2007; Piotti et al., 2014; Xu-Dubois et al., 2016). Nevertheless, it is a type III intermediate filament protein, expressed ubiquitously in mesenchymal cells and involved endothelial cell adhesion (Dave and Bayless, 2014). Hence its targeting by humoral autoimmunity in accelerating allograft vasculopathy is credible.

Furthermore, a number of other auto-antigens have been suggested to be associated with AV like autoantibodies to cardiac myosin (Kalache et al., 2011), collagen V (Tiriveedhi et al., 2013) and perlecan (Cardinal et al., 2013); however more extensive work is required to validate which, if any, of the autoantibody or autoantibodies are pertinent for vasculopathy progression.

The demonstration of development of allograft vasculopathy in parental allograft which were transplanted into F1 hybrid recipients provides the strongest evident for humoral autoimmunity contributing to progression of AV independent of adaptive alloimmune response. As in bm12 to BL6 model, in this parental to F1 model, development of GC autoimmunity was dependent on passenger donor CD4 T cells in donor heart graft. Interestingly, the donor grafts remained disease free when transplanted into SAP^{-ve}.F1 hybrid recipients (figure 5.10d). Although our recent work has shown that passenger CD4 T cells can provide help to host alloreactive B cells for generating alloantibody against MHC alloantigen expressed on donor cells (Harper et al., 2016), however alloantibody was not generated in the F1 recipients in this model, most likely because, as the H-2^{b} and H-2^{d} MHC antigens are membrane proteins, hence they provoke robust, central deletional B cell tolerance (Hartley et al., 1991) (figure 5.10b)

Despite the fact that robust GC autoimmunity is generated in the recipients in bm12 to BL6 and parental to F1 hybrid model, the autoimmune disease manifestations were not observed in our recipients' native organs (Harper et al., 2016; Motallebzadeh et al., 2012). This is interesting in the light of Eisenberg work, who have used this as a model of systemic lupus erythematosus (SLE) (Eisenberg and Via, 2012). However, our group has noticed occasional skin and gut manifestations consistent with autoimmune disease in long term surviving recipients (more than 6 months). Interestingly, in my work, of the group which developed early and augmented autoantibodies and accelerated rejection following challenge with primed bm12 allografts, 2 of 5 recipients had to be culled early due to the development of similar features. Thus it appears that the level of autoantibody was not high enough in our WT recipients of bm12 heart grafts to provoke systemic autoimmune disease and it is likely that in other studies where it was used as a model of SLE, the number of cells and type of cells transferred were different than in our studies. Hence it is likely that the development of SLE like disease depends on the number and type of donor cells which were

transferred into recipients. Further experiments are needed to check firstly how many CD4 T cells were present in donor hearts when harvested from bm12 donor grafts and secondly to examine the development of autoantibodies in the recipients after adoptive transfer of different number of CD4 T cells to identify number of CD4 T cells required to trigger autoimmunity in the recipients and systemic manifestation of autoimmune diseases.

Although one of the recent studies has shown the development of native arterial disease in BL6 recipients of bm12 heart grafts, this was shown only in atherosclerosis-prone mice (apoE^{-ve}) (Zhou et al., 2015) and autoantibodies were not examined in this study. But our findings suggest that they would have developed autoantibodies and it is likely that those autoantibodies may be contributing to native arteriopathy. This would be consistent with a two-hit hypothesis, whereby the potential for autoantibody to affect arterial disease is greatly enhanced by concurrent damage that possibly exposes previously sequestered target autoantigen (Win and Pettigrew, 2010). The insult to the sequestered self-antigens for their exposure or release may not be the antigen non-specific ischaemic reperfusion injury as in our experiment which showed that the syngeneic donor heart grafts remained disease free that had been simultaneously primed for humoral autoimmunity (figure 5.8e).

A possible alternative is that allograft damage is initiated by innate immune recognition of non-self allo-antigen either by monocytes/macrophages (Chow et al., 2016; Oberbarnscheidt et al., 2014; Zecher et al., 2009) or NK cells (Kroemer et al., 2008). Recipient's monocytes can respond to allogenic non-self-determinants like polymorphism in alpha signal regulatory protein (α SIRP) (Dai et al., 2017) on donors. In contrast, MHC incompatibility can result in alloreactive NK cells, which may be cytolytic to allogeneic cells (Kroemer et al., 2008). Uehara et al (Uehara et al., 2005) have also reported that parental allografts did not reject when transplanted into NK-cell depleted F1 recipients. This is in consistent with our findings as well. However, with the evidence of development of GC autoimmunity in F1 recipients of parental allografts (figure 5.10), the requirement for NK cells in rejection of parental heart allografts most likely reflects Fc-receptor mediated recognition of bound autoantibody on graft endothelium. Although the underlying mechanism remain unclear, these autoantibodies responses may synergise or partially dependent upon simultaneous complement activation (Yin et al., 2004). This is consistent

with our findings that complement deposition was not observed within allografts explanted from NK-cell depleted recipients (figure 5.10) who remained disease free as well.

Implications of this work

The implications for this work is that bm12 to BL6 model remains the most widely studied experimental model which has minimal mismatch in MHC alloantigens between donor and recipients which is related with clinical transplantation as every effort is made in clinical transplantation to minimise the mismatch. However, doing so, there is a possibility that donor CD4 T cells survive long enough to trigger autoimmunity in recipients through GVH recognition. This is consistent with the 'passenger lymphocyte syndrome' seen in recipients of solid organ allografts and is increasingly emphasized (Nadarajah et al., 2013; Turner et al., 2014). To what extent this donor induced autoimmunity in recipients contribute to allograft rejection remains unknown. However, our study demonstrated that donor induced autoimmunity in the recipients mediates progression of allograft vasculopathy independent of adaptive alloimmune responses. We have shown that irrespective of the mechanisms and factors which initiate this autoimmunity in recipients, recipient's T_{FH} CD4 T cells help B cells to differentiate into GCs resulting in long term autoimmunity and has deleterious impacts on allograft vasculopathy. Development of this TAA in human transplant recipients would likely have detrimental impact on transplant outcomes. This is particularly relevant to the emerging evidence to develop strategies that aim to promote transplant tolerance in an attempt to prolong graft survival by achieving mixed haematopoietic chimerism (Li and Sykes, 2012), as in our experiments with mixed bone marrow chimeric recipients in which donor passenger CD4 T cells in allografts were capable of breaking the established tolerance to alloantigens in recipients and resulted in development of long lasting autoantibodies. Development of this long lasting autoimmunity effects allografts deleteriously. These findings are consistent with the previous murine study by Shinoda et al (Shinoda et al., 2014) in which depletion of both donor and recipient Foxp3^{+ve}.T-regs cells resulted in allograft rejection despite persistence of chimerism. Hence, one should be cautious of the development of autoimmunity while achieving tolerance through mixed haematopoietic chimerism. Hence, modulation of donor related factors should be considered in developing therapeutic approaches to achieve tolerance in recipients like modulating the donor lymphocyte fraction prior to implantation during ex-vivo normothermic donor organ perfusion.

As the development of these autoantibodies was initiated by donor CD4 T cells in this model but maintained by recipient CD4 T cells through germinal centres, manipulation of various pathways on this subset of CD4 T cells may prolong graft survival. In line with this, inhibition of SAP signaling in recipients prolonged allograft survival significantly (figure 5.5). Similarly, antibiotic treatment of both donor and recipients prolonged skin grafts in minimal mismatch model by modulating composition of microbiota (Lei et al., 2016). Kim et al have shown amelioration of antibody mediated rejection by blocking germinal centres through co-stimulation blockade in nonhuman primate kidney transplant model (Kim et al., 2014). Vincenti et al have shown improved kidney graft and patient survival in human kidney transplant recipients by selectively blocking the activated CD4 T cells via CTLA4 pathway (Vincenti, 2016).

Finally my findings have wider implications in understanding the underlying mechanisms responsible for development of autoimmune phenomena. The experiments in which SAP signalling was inhibited in donor bm12 CD4 T cells which were incapable of differentiation into long term T_{FH} cells are nevertheless the key trigger for generating long lasting humoral autoimmunity. Furthermore bm12K^d transfer experiments confirming that late help to follicular B cells is being provided by the naïve host CD4 T cells which differentiate into long term T_{FH} cell for development of long lasting autoimmunity; suggest that at the time of autoimmune disease manifestations the trigger for the autoimmunity like any viral challenge or environmental factors may not be present but there may be a different factor which may be maintaining the disease process. This is very pertinent in idiopathic immunological disorders as the triggering factor for these diseases may not be identifiable at the time of symptoms hence understanding of the underlying mechanism for these autoimmune diseases would be helpful in devising therapeutic strategies. Likewise in my work, although donor CD4 T cells triggered autoimmunity in recipients, they could not maintain it for long term and it was the recipients T_{FH} cell which maintained and propagated the donor induced auto-immunity in the recipient through long term GCs; developing selective strategies to target the pathogenic population of T_{FH} cells may be a future avenue to deal with different autoimmune diseases.

Limitations of this work

It is important to highlight the limitations of this work that in some of the experiments the experimental number of mice was small and there was lack of appropriate control for each set of experiment. However, results from each set of experiment was cross examined by multiple modalities and in different group of animals to overcome this deficit and to confirm the findings. Furthermore I agree that this deficiency can be avoided in future work by designing experiment which should be more focused with appropriate numbers, good planning and possibly using little may control for each set of experiments. With respect to bm12 to BL6 model, potential mutations in using the inbred bm12 mice was clearly a point of concern and should have used the commercially available bm12 mice to avoid this problem. However, this strain was genotyped regularly and compared to the commercially available bm12 strain for any potential mutations.

References

Abbey, J.L., and H.C. O'Neill. 2008. Expression of T-cell receptor genes during early T-cell development. *Immunol Cell Biol* 86:166-174.

Alfonso, C., J.O. Han, G.S. Williams, and L. Karlsson. 2001. The impact of H2-DM on humoral immune responses. *J Immunol* 167:6348-6355.

Ali, J.M., E.M. Bolton, J.A. Bradley, and G.J. Pettigrew. 2013. Allorecognition pathways in transplant rejection and tolerance. *Transplantation* 96:681-688.

Ali, J.M., M.C. Negus, T.M. Conlon, I.G. Harper, M.S. Qureshi, R. Motallebzadeh, R. Willis, K. Saeb-Parsy, E.M. Bolton, J.A. Bradley, and G.J. Pettigrew. 2016. Diversity of the CD4 T Cell Alloresponse: The Short and the Long of It. *Cell reports* 14:1232-1245.

Allen, C.D., T. Okada, and J.G. Cyster. 2007a. Germinal-center organization and cellular dynamics. *Immunity* 27:190-202.

Allen, C.D., T. Okada, H.L. Tang, and J.G. Cyster. 2007b. Imaging of germinal center selection events during affinity maturation. *Science* 315:528-531.

Alsughayyir, J., M. Chhabra, M.S. Qureshi, M. Mallik, J.M. Ali, I. Gamper, E.L. Moseley, S. Peacock, V. Kosmoliaptsis, M.J. Goddard, M.A. Linterman, R. Motallebzadeh, and G.J. Pettigrew. 2018. Relative Frequencies of Alloantigen-Specific Helper CD4 T Cells and B Cells Determine Mode of Antibody-Mediated Allograft Rejection. *Front Immunol* 9:3039.

Amico, P., G. Honger, D. Bielmann, D. Lutz, D. Garzoni, J. Steiger, M.J. Mihatsch, D. Dragun, and S. Schaub. 2008. Incidence and prediction of early antibody-mediated rejection due to non-human leukocyte antigen-antibodies. *Transplantation* 85:1557-1563.

Anderson, G., and Y. Takahama. 2012. Thymic epithelial cells: working class heroes for T cell development and repertoire selection. *Trends Immunol* 33:256-263.

Anderton, S.M. 2004. Post-translational modifications of self antigens: implications for autoimmunity. *Current opinion in immunology* 16:753-758.

Anderton, S.M., and D.C. Wraith. 2002. Selection and fine-tuning of the autoimmune T-cell repertoire. *Nat Rev Immunol* 2:487-498.

Angaswamy, N., C. Klein, V. Tiriveedhi, J. Gaut, S. Anwar, A. Rossi, D. Phelan, J.R. Wellen, S. Shenoy, W.C. Chapman, and T. Mohanakumar. 2014. Immune responses to collagen-IV and fibronectin in renal transplant recipients with transplant glomerulopathy. *Am J Transplant* 14:685-693.

Aranda, J.M., Jr., and J. Hill. 2000. Cardiac transplant vasculopathy. *Chest* 118:1792-1800.

Ardehali, A., M.P. Fischbein, J. Yun, Y. Irie, M.C. Fishbein, and H. Laks. 2002. Indirect alloreactivity and chronic rejection. *Transplantation* 73:1805-1807.

Azimzadeh, A.M., S. Pfeiffer, G.S. Wu, C. Schroder, H. Zhou, G.L. Zorn, 3rd, M. Kehry, G.G. Miller, M.L. Rose, and R.N. Pierson, 3rd. 2005. Humoral immunity to vimentin is associated with cardiac allograft injury in nonhuman primates. *Am J Transplant* 5:2349-2359.

Bach, F., and K. Hirschhorn. 1964. Lymphocyte Interaction: A Potential Histocompatibility Test in Vitro. *Science* 143:813-814.

Bachmann, M.F., B. Odermatt, H. Hengartner, and R.M. Zinkernagel. 1996. Induction of long-lived germinal centers associated with persisting antigen after viral infection. *J Exp Med* 183:2259-2269.

Baker, R.J., M.P. Hernandez-Fuentes, P.A. Brookes, A.N. Chaudhry, H.T. Cook, and R.I. Lechler. 2001a. Loss of Direct and Maintenance of Indirect Alloresponses in Renal Allograft Recipients: Implications for the Pathogenesis of Chronic Allograft Nephropathy. *The Journal of Immunology* 167:7199-7206.

Baker, R.J., M.P. Hernandez-Fuentes, P.A. Brookes, A.N. Chaudhry, H.T. Cook, and R.I. Lechler. 2001b. Loss of direct and maintenance of indirect alloresponses in renal allograft recipients: implications for the pathogenesis of chronic allograft nephropathy. *J Immunol* 167:7199-7206.

Baniyash, M. 2004. TCR zeta-chain downregulation: curtailing an excessive inflammatory immune response. *Nat Rev Immunol* 4:675-687.

Benichou, G., A. Alessandrini, R.S. Charrad, and D.S. Wilkes. 2007. Induction of autoimmunity after allotransplantation. *Front Biosci* 12:4362-4369.

Berek, C., A. Berger, and M. Apel. 1991. Maturation of the immune response in germinal centers. *Cell* 67:1121-1129.

Bergqvist, P., A. Stensson, L. Hazanov, A. Holmberg, J. Mattsson, R. Mehr, M. Bemark, and N.Y. Lycke. 2013. Re-utilization of germinal centers in multiple Peyer's patches results in highly synchronized, oligoclonal, and affinity-matured gut IgA responses. *Mucosal Immunol* 6:122-135.

Berland, R., and H.H. Wortis. 2002. Origins and functions of B-1 cells with notes on the role of CD5. *Annu Rev Immunol* 20:253-300.

Billingham, M.E. 1992. Histopathology of graft coronary disease. *J Heart Lung Transplant* 11:S38-44.

Bjorkman, P.J., M.A. Saper, B. Samraoui, W.S. Bennett, J.L. Strominger, and D.C. Wiley. 1987. Structure of the human class I histocompatibility antigen, HLA-A2. *Nature* 329:506-512.

Blink, E.J., A. Light, A. Kallies, S.L. Nutt, P.D. Hodgkin, and D.M. Tarlinton. 2005. Early appearance of germinal center-derived memory B cells and plasma cells in blood after primary immunization. *J Exp Med* 201:545-554.

Bordron, A., M. Dueymes, Y. Levy, C. Jamin, J.P. Leroy, J.C. Piette, Y. Shoenfeld, and P.Y. Youinou. 1998. The binding of some human antiendothelial cell antibodies induces endothelial cell apoptosis. *J Clin Invest* 101:2029-2035.

Brard, F., M. Shannon, E.L. Prak, S. Litwin, and M. Weigert. 1999. Somatic mutation and light chain rearrangement generate autoimmunity in anti-single-stranded DNA transgenic MRL/lpr mice. *J Exp Med* 190:691-704.

Breitfeld, D., L. Ohl, E. Kremmer, J. Ellwart, F. Sallusto, M. Lipp, and R. Forster. 2000. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med* 192:1545-1552.

Bretscher, P., and M. Cohn. 1970. A theory of self-nonself discrimination. *Science* 169:1042-1049.

Brink, R. 2014. The imperfect control of self-reactive germinal center B cells. *Curr Opin Immunol* 28:97-101.

Brouard, S., E. Mansfield, C. Braud, L. Li, M. Giral, S.C. Hsieh, D. Baeten, M. Zhang, J. Ashton-Chess, C. Braudeau, F. Hsieh, A. Dupont, A. Pallier, A. Moreau, S. Louis, C. Ruiz, O. Salvatierra, J.P. Soulillou, and M. Sarwal. 2007. Identification of a peripheral blood transcriptional biomarker panel associated with operational renal allograft tolerance. *Proc Natl Acad Sci U S A* 104:15448-15453.

Brunn, G.J., S. Saadi, and J.L. Platt. 2006. Differential regulation of endothelial cell activation by complement and interleukin 1alpha. *Circ Res* 98:793-800.

Burlingham, W.J., R.B. Love, E. Jankowska-Gan, L.D. Haynes, Q. Xu, J.L. Bobadilla, K.C. Meyer, M.S. Hayney, R.K. Braun, D.S. Greenspan, B. Gopalakrishnan, J. Cai, D.D. Brand, S. Yoshida, O.W. Cummings, and D.S. Wilkes. 2007. IL-17-dependent cellular immunity to collagen type V predisposes to obliterative bronchiolitis in human lung transplants. *J Clin Invest* 117:3498-3506.

Busser, B.W., B.S. Adair, J. Erikson, and T.M. Laufer. 2003. Activation of diverse repertoires of autoreactive T cells enhances the loss of anti-dsDNA B cell tolerance. *J Clin Invest* 112:1361-1371.

Butcher, E.C., R.V. Rouse, R.L. Coffman, C.N. Nottenburg, R.R. Hardy, and I.L. Weissman. 1982. Surface phenotype of Peyer's patch germinal center cells: implications for the role of germinal centers in B cell differentiation. *J Immunol* 129:2698-2707.

Callaghan, C.J., F.J. Rouhani, M.C. Negus, A.J. Curry, E.M. Bolton, J.A. Bradley, and G.J. Pettigrew. 2007. Abrogation of antibody-mediated allograft rejection by regulatory CD4 T cells with indirect allospecificity. *J Immunol* 178:2221-2228.

Cardinal, H., M. Dieude, N. Brassard, S. Qi, N. Patey, M. Soulez, D. Beillevaire, F. Echeverry, C. Daniel, Y. Durocher, F. Madore, and M.J. Hebert. 2013. Antiperlecan antibodies are novel accelerators of immune-mediated vascular injury. *Am J Transplant* 13:861-874.

Carding, S.R., and P.J. Egan. 2002. Gammadelta T cells: functional plasticity and heterogeneity. *Nat Rev Immunol* 2:336-345.

Cervenak, L., A. Magyar, R. Boja, and G. Laszlo. 2001. Differential expression of GL7 activation antigen on bone marrow B cell subpopulations and peripheral B cells. *Immunol Lett* 78:89-96.

Chan, W.F., H. Razavy, B. Luo, A.M. Shapiro, and C.C. Anderson. 2008. Development of either split tolerance or robust tolerance along with humoral tolerance to donor and third-party alloantigens in nonmyeloablative mixed chimeras. *J Immunol* 180:5177-5186.

Chen, C., Z. Nagy, E.L. Prak, and M. Weigert. 1995. Immunoglobulin heavy chain gene replacement: a mechanism of receptor editing. *Immunity* 3:747-755.

Chen, C.A., and H. Okayama. 1988. Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA. *Biotechniques* 6:632-638.

Chen, F., M.A. Maldonado, M. Madaio, and R.A. Eisenberg. 1998. The role of host (endogenous) T cells in chronic graft-versus-host autoimmune disease. *J Immunol* 161:5880-5885.

Chhabra, M., J. Alsughayyir, M.S. Qureshi, M. Mallik, J.M. Ali, I. Gamper, E.L. Moseley, S. Peacock, V. Kosmoliaptsis, M.J. Goddard, M.A. Linterman, R. Motallebzadeh, and G.J. Pettigrew. 2018. Germinal Center Alloantibody Responses Mediate Progression of Chronic Allograft Injury. *Front Immunol* 9:3038.

Choudhury, A., M.A. Maldonado, P.L. Cohen, and R.A. Eisenberg. 2005. The role of host CD4 T cells in the pathogenesis of the chronic graft-versus-host model of systemic lupus erythematosus. *J Immunol* 174:7600-7609.

Chow, K.V., R.B. Delconte, N.D. Huntington, D.M. Tarlinton, R.M. Sutherland, Y. Zhan, and A.M. Lew. 2016. Innate Allorecognition Results in Rapid Accumulation of Monocyte-Derived Dendritic Cells. *J Immunol* 197:2000-2008.

Chung, Y., S.Y. Ko, H.J. Ko, and C.Y. Kang. 2005. Split peripheral tolerance: CD40 ligation blocks tolerance induction for CD8 T cells but not for CD4 T cells in response to intestinal antigens. *Eur J Immunol* 35:1381-1390.

Ciubotariu, R., Z. Liu, A.I. Colovai, E. Ho, S. Itescu, S. Ravalli, M.A. Hardy, R. Cortesini, E.A. Rose, and N. Suciu-Foca. 1998. Persistent allopeptide reactivity and epitope spreading in chronic rejection of organ allografts. *J Clin Invest* 101:398-405.

Coico, R.F., B.S. Bhogal, and G.J. Thorbecke. 1983. Relationship of germinal centers in lymphoid tissue to immunologic memory. VI. Transfer of B cell memory with lymph node cells fractionated according to their receptors for peanut agglutinin. *J Immunol* 131:2254-2257.

Collins, A.B., E.E. Schneeberger, M.A. Pascual, S.L. Saidman, W.W. Williams, N. Tolkoff-Rubin, A.B. Cosimi, and R.B. Colvin. 1999. Complement activation in acute humoral renal allograft rejection: diagnostic significance of C4d deposits in peritubular capillaries. *J Am Soc Nephrol* 10:2208-2214.

Conlon, T.M., K. Saeb-Parsy, J.L. Cole, R. Motallebzadeh, M.S. Qureshi, S. Rehakova, M.C. Negus, C.J. Callaghan, E.M. Bolton, J.A. Bradley, and G.J. Pettigrew. 2012. Germinal center alloantibody responses are mediated exclusively by indirect-pathway CD4 T follicular helper cells. *J Immunol* 188:2643-2652.

Cooper, M.D., R.D. Peterson, and R.A. Good. 1965. Delineation of the Thymic and Bursal Lymphoid Systems in the Chicken. *Nature* 205:143-146.

Corry, R.J., H.J. Winn, and P.S. Russell. 1973. Primarily vascularized allografts of hearts in mice. The role of H-2D, H-2K, and non-H-2 antigens in rejection. *Transplantation* 16:343-350.

Coupel, S., F. Leboeuf, G. Boulday, J.P. Soulillou, and B. Charreau. 2004. RhoA activation mediates phosphatidylinositol 3-kinase-dependent proliferation of human vascular endothelial cells: an alloimmune mechanism of chronic allograft nephropathy. *J Am Soc Nephrol* 15:2429-2439.

Crotty, S. 2011. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol* 29:621-663.

Crotty, S. 2014. T follicular helper cell differentiation, function, and roles in disease. *Immunity* 41:529-542.

Crotty, S. 2015. A brief history of T cell help to B cells. *Nat Rev Immunol* 15:185-189.

Crotty, S., R.J. Johnston, and S.P. Schoenberger. 2010. Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation. *Nat Immunol* 11:114-120.

Crotty, S., E.N. Kersh, J. Cannons, P.L. Schwartzberg, and R. Ahmed. 2003. SAP is required for generating long-term humoral immunity. *Nature* 421:282-287.

Cruickshank, M.N., and D. Ulgiati. 2010. The role of notch signaling in the development of a normal B-cell repertoire. *Immunol Cell Biol* 88:117-124.

Cunningham, A.F., A. Flores-Langarica, S. Bobat, C.C. Dominguez Medina, C.N. Cook, E.A. Ross, C. Lopez-Macias, and I.R. Henderson. 2014. B1b cells recognize protective antigens after natural infection and vaccination. *Front Immunol* 5:535.

Curotto de Lafaille, M.A., and J.J. Lafaille. 2009. Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor? *Immunity* 30:626-635.

Cyster, J.G. 1999. Chemokines and cell migration in secondary lymphoid organs. *Science* 286:2098-2102.

Czar, M.J., E.N. Kersh, L.A. Mijares, G. Lanier, J. Lewis, G. Yap, A. Chen, A. Sher, C.S. Duckett, R. Ahmed, and P.L. Schwartzberg. 2001. Altered lymphocyte responses and cytokine

production in mice deficient in the X-linked lymphoproliferative disease gene SH2D1A/DSHP/SAP. *Proc Natl Acad Sci U S A* 98:7449-7454.

Dai, H., A.J. Friday, K.I. Abou-Daya, A.L. Williams, S. Mortin-Toth, M.L. Nicotra, D.M. Rothstein, W.D. Shlomchik, T. Matozaki, J.S. Isenberg, M.H. Oberbarnscheidt, J.S. Danska, and F.G. Lakkis. 2017. Donor SIRPalpha polymorphism modulates the innate immune response to allogeneic grafts. *Science immunology* 2:

Dave, J.M., and K.J. Bayless. 2014. Vimentin as an integral regulator of cell adhesion and endothelial sprouting. *Microcirculation (New York, N.Y. : 1994)* 21:333-344.

Davidson, A. 2014. Editorial: autoimmunity to vimentin and lupus nephritis. *Arthritis Rheumatol* 66:3251-3254.

de Mestre, A., L. Noronha, B. Wagner, and D.F. Antczak. 2010. Split immunological tolerance to trophoblast. *Int J Dev Biol* 54:445-455.

de Vinuesa, C.G., M.C. Cook, J. Ball, M. Drew, Y. Sunners, M. Cascalho, M. Wabl, G.G. Klaus, and I.C. MacLennan. 2000. Germinal centers without T cells. *J Exp Med* 191:485-494.

Deenick, E.K., A. Chan, C.S. Ma, D. Gatto, P.L. Schwartzberg, R. Brink, and S.G. Tangye. 2010. Follicular helper T cell differentiation requires continuous antigen presentation that is independent of unique B cell signaling. *Immunity* 33:241-253.

Doyle, H.A., and M.J. Mamula. 2012. Autoantigenesis: the evolution of protein modifications in autoimmune disease. *Curr Opin Immunol* 24:112-118.

Dragun, D., R. Catar, and A. Philippe. 2013. Non-HLA antibodies in solid organ transplantation: recent concepts and clinical relevance. *Curr Opin Organ Transplant* 18:430-435.

Dragun, D., R. Catar, and A. Philippe. 2016. Non-HLA antibodies against endothelial targets bridging allo- and autoimmunity. *Kidney Int* 90:280-288.

Dragun, D., D.N. Muller, J.H. Brasen, L. Fritsche, M. Nieminen-Kelha, R. Dechend, U. Kintscher, B. Rudolph, J. Hoebeke, D. Eckert, I. Mazak, R. Plehm, C. Schonemann, T. Unger, K. Budde, H.H. Neumayer, F.C. Luft, and G. Wallukat. 2005. Angiotensin II type 1-receptor activating antibodies in renal-allograft rejection. *N Engl J Med* 352:558-569.

Eisenberg, R.A., and C.S. Via. 2012. T cells, murine chronic graft-versus-host disease and autoimmunity. *J Autoimmun* 39:240-247.

Eng, H.S., G. Bennett, S.H. Chang, H. Dent, S.P. McDonald, P. Bardy, P. Coghlan, G.R. Russ, and P.T. Coates. 2011. Donor HLA Specific Antibodies Predict Development and Define Prognosis in Transplant Glomerulopathy. *Hum Immunol*

Faro, J., and M. Or-Guil. 2013. How oligoclonal are germinal centers? A new method for estimating clonal diversity from immunohistological sections. *BMC Bioinformatics* 14 Suppl 6:S8.

Fatenejad, S., M.J. Mamula, and J. Craft. 1993. Role of intermolecular/intrastructural B- and T-cell determinants in the diversification of autoantibodies to ribonucleoprotein particles. *Proc Natl Acad Sci U S A* 90:12010-12014.

Fazilleau, N., L.J. McHeyzer-Williams, H. Rosen, and M.G. McHeyzer-Williams. 2009. The function of follicular helper T cells is regulated by the strength of T cell antigen receptor binding. *Nat Immunol* 10:375-384.

Fedoseyeva, E.V., K. Kishimoto, H.K. Rolls, B.M. Illigens, V.M. Dong, A. Valujskikh, P.S. Heeger, M.H. Sayegh, and G. Benichou. 2002. Modulation of tissue-specific immune response to cardiac myosin can prolong survival of allogeneic heart transplants. *J Immunol* 169:1168-1174.

Fedoseyeva, E.V., R.C. Tam, I.A. Popov, P.L. Orr, M.R. Garovoy, and G. Benichou. 1996. Induction of T cell responses to a self-antigen following allotransplantation. *Transplantation* 61:679-683.

Fedoseyeva, E.V., F. Zhang, P.L. Orr, D. Levin, H.J. Buncke, and G. Benichou. 1999. De novo autoimmunity to cardiac myosin after heart transplantation and its contribution to the rejection process. *J Immunol* 162:6836-6842.

Fehling, H.J., A. Krotkova, C. Saint-Ruf, and H. von Boehmer. 1995. Crucial role of the pre-T-cell receptor alpha gene in development of alpha beta but not gamma delta T cells. *Nature* 375:795-798.

Ferrer, I.R., J. Hester, A. Bushell, and K.J. Wood. 2014. Induction of transplantation tolerance through regulatory cells: from mice to men. *Immunol Rev* 258:102-116.

Feucht, H.E., E. Felber, M.J. Gokel, G. Hillebrand, U. Nattermann, C. Brockmeyer, E. Held, G. Riethmüller, W. Land, and E. Albert. 1991. Vascular deposition of complement-split products in kidney allografts with cell-mediated rejection. *Clin Exp Immunol* 86:464-470.

Fischbein, M.P., A. Ardehali, J. Yun, S. Schoenberger, H. Laks, Y. Irie, P. Dempsey, G. Cheng, M.C. Fishbein, and B. Bonavida. 2000. CD40 signaling replaces CD4+ lymphocytes and its blocking prevents chronic rejection of heart transplants. *J Immunol* 165:7316-7322.

Fischbein, M.P., J. Yun, H. Laks, Y. Irie, M.C. Fishbein, M. Espejo, B. Bonavida, and A. Ardehali. 2001. CD8+ lymphocytes augment chronic rejection in a MHC class II mismatched model. *Transplantation* 71:1146-1153.

Fishelson, Z., G. Attali, and D. Mevorach. 2001. Complement and apoptosis. *Mol Immunol* 38:207-219.

Ford, M.S., K.J. Young, Z. Zhang, P.S. Ohashi, and L. Zhang. 2002. The immune regulatory function of lymphoproliferative double negative T cells in vitro and in vivo. *J Exp Med* 196:261-267.

Fukami, N., S. Ramachandran, D. Saini, M. Walter, W. Chapman, G.A. Patterson, and T. Mohanakumar. 2009. Antibodies to MHC class I induce autoimmunity: role in the pathogenesis of chronic rejection. *J Immunol* 182:309-318.

Furukawa, Y., S.E. Cole, R.V. Shah, Y. Fukumoto, P. Libby, and R.N. Mitchell. 2004. Wild-type but not interferon-gamma-deficient T cells induce graft arterial disease in the absence of B cells. *Cardiovasc Res* 63:347-356.

Gao, S.Z., E.L. Alderman, J.S. Schroeder, J.F. Silverman, and S.A. Hunt. 1988. Accelerated coronary vascular disease in the heart transplant patient: coronary arteriographic findings. *J Am Coll Cardiol* 12:334-340.

Gao, W., P.S. Topham, J.A. King, S.T. Smiley, V. Csizmadia, B. Lu, C.J. Gerard, and W.W. Hancock. 2000. Targeting of the chemokine receptor CCR1 suppresses development of acute and chronic cardiac allograft rejection. *J Clin Invest* 105:35-44.

Garside, P., E. Ingulli, R.R. Merica, J.G. Johnson, R.J. Noelle, and M.K. Jenkins. 1998. Visualization of specific B and T lymphocyte interactions in the lymph node. *Science* 281:96-99.

Gasque, P., S.K. Singhrao, J.W. Neal, O. Gotze, and B.P. Morgan. 1997. Expression of the receptor for complement C5a (CD88) is up-regulated on reactive astrocytes, microglia, and endothelial cells in the inflamed human central nervous system. *Am J Pathol* 150:31-41.

Gaston, R.S., J.M. Cecka, B.L. Kasiske, A.M. Fieberg, R. Leduc, F.C. Cosio, S. Gourishankar, J. Grande, P. Halloran, L. Hunsicker, R. Mannon, D. Rush, and A.J. Matas. 2010. Evidence

for antibody-mediated injury as a major determinant of late kidney allograft failure. *Transplantation* 90:68-74.

Gavioli, R., Q.J. Zhang, M. Marastoni, R. Guerrini, E. Reali, R. Tomatis, M.G. Masucci, and S. Traniello. 1995. Effect of anchor residue modifications on the stability of HLA-A11/peptide complexes. *Biochem Biophys Res Commun* 206:8-14.

Gerdes, T., and M. Wabl. 2004. Autoreactivity and allelic inclusion in a B cell nuclear transfer mouse. *Nat Immunol* 5:1282-1287.

Girnita, A.L., K.R. McCurry, and A. Zeevi. 2007. Increased lung allograft failure in patients with HLA-specific antibody. *Clin Transpl* 231-239.

Glysing-Jensen, T., A. Raisanen-Sokolowski, M.H. Sayegh, and M.E. Russell. 1997. Chronic blockade of CD28-B7-mediated T-cell costimulation by CTLA4Ig reduces intimal thickening in MHC class I and II incompatible mouse heart allografts. *Transplantation* 64:1641-1645.

Goers, T.A., S. Ramachandran, A. Aloush, E. Trulock, G.A. Patterson, and T. Mohanakumar. 2008. De novo production of K-alpha1 tubulin-specific antibodies: role in chronic lung allograft rejection. *J Immunol* 180:4487-4494.

Gonzalez, M., R. Merino, A.L. Gonzalez, and J. Merino. 1995. The ability of B cells to participate in allogeneic cognate T-B cell interactions in vitro depends on the presence of CD4+ T cells during their development. *J Immunol* 155:1091-1100.

Good-Jacobson, K.L., C.G. Szumilas, L. Chen, A.H. Sharpe, M.M. Tomayko, and M.J. Shlomchik. 2010. PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. *Nat Immunol* 11:535-542.

Gowans, J.L., G.D. Mc, and D.M. Cowen. 1962. Initiation of immune responses by small lymphocytes. *Nature* 196:651-655.

Graham, F.L., and A.J. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52:456-467.

Graham, J.A., R.A. Wilkinson, T. Hirohashi, C.M. Chase, R.B. Colvin, J.C. Madsen, J.A. Fishman, and P.S. Russell. 2009. Viral infection induces de novo lesions of coronary allograft vasculopathy through a natural killer cell-dependent pathway. *Am J Transplant* 9:2479-2484.

Gratwohl, A., B. Dohler, M. Stern, and G. Opelz. 2008. H-Y as a minor histocompatibility antigen in kidney transplantation: a retrospective cohort study. *Lancet* 372:49-53.

Hamaguchi, Y., Y. Xiu, K. Komura, F. Nimmerjahn, and T.F. Tedder. 2006. Antibody isotype-specific engagement of Fcgamma receptors regulates B lymphocyte depletion during CD20 immunotherapy. *J Exp Med* 203:743-753.

Han, S., K. Hathcock, B. Zheng, T.B. Kepler, R. Hodes, and G. Kelsoe. 1995. Cellular interaction in germinal centers. Roles of CD40 ligand and B7-2 in established germinal centers. *J Immunol* 155:556-567.

Han, S., B. Zheng, D.G. Schatz, E. Spanopoulou, and G. Kelsoe. 1996. Neoteny in lymphocytes: Rag1 and Rag2 expression in germinal center B cells. *Science* 274:2094-2097.

Hansen, T.H., and H.Y. Tse. 1987. Insights into immune-response gene function using an Ia mutant mouse strain. *Crit Rev Immunol* 7:169-191.

Haque, M.A., T. Mizobuchi, K. Yasufuku, T. Fujisawa, R.R. Brutkiewicz, Y. Zheng, K. Woods, G.N. Smith, O.W. Cummings, K.M. Heidler, J.S. Blum, and D.S. Wilkes. 2002. Evidence for immune responses to a self-antigen in lung transplantation: role of type V collagen-specific T cells in the pathogenesis of lung allograft rejection. *J Immunol* 169:1542-1549.

Hardy, R.R., and K. Hayakawa. 2001. B cell development pathways. *Annu Rev Immunol* 19:595-621.

Harper, I.G., J.M. Ali, S.J. Harper, E. Wlodek, J. Alsughayyir, M.C. Negus, M.S. Qureshi, R. Motalleb-Zadeh, K. Saeb-Parsy, E.M. Bolton, J.A. Bradley, M.R. Clatworthy, T.M. Conlon, and G.J. Pettigrew. 2016. Augmentation of Recipient Adaptive Alloimmunity by Donor Passenger Lymphocytes within the Transplant. *Cell reports* 15:1214-1227.

Harrison, J.H., J.P. Merrill, and J.E. Murray. 1956. Renal homotransplantation in identical twins. *Surg Forum* 6:432-436.

Hartley, S.B., J. Crosbie, R. Brink, A.B. Kantor, A. Basten, and C.C. Goodnow. 1991. Elimination from peripheral lymphoid tissues of self-reactive B lymphocytes recognizing membrane-bound antigens. *Nature* 353:765-769.

Hasegawa, T., S.H. Visovatti, M.C. Hyman, T. Hayasaki, and D.J. Pinsky. 2007. Heterotopic vascularized murine cardiac transplantation to study graft arteriopathy. *Nat Protoc* 2:471-480.

Hattori, T., H. Shimokawa, M. Higashi, J. Hiroki, Y. Mukai, K. Kaibuchi, and A. Takeshita. 2004. Long-term treatment with a specific Rho-kinase inhibitor suppresses cardiac allograft vasculopathy in mice. *Circ Res* 94:46-52.

Haurum, J.S., L. Tan, G. Arsequell, P. Frodsham, A.C. Lellouch, P.A. Moss, R.A. Dwek, A.J. McMichael, and T. Elliott. 1995. Peptide anchor residue glycosylation: effect on class I major histocompatibility complex binding and cytotoxic T lymphocyte recognition. *Eur J Immunol* 25:3270-3276.

Haynes, L.D., E. Jankowska-Gan, A. Sheka, M.R. Keller, M.P. Hernandez-Fuentes, R.I. Lechler, V. Seyfert-Margolis, L.A. Turka, K.A. Newell, and W.J. Burlingham. 2012. Donor-specific indirect pathway analysis reveals a B-cell-independent signature which reflects outcomes in kidney transplant recipients. *Am J Transplant* 12:640-648.

Heeger, P.S., P.N. Lalli, F. Lin, A. Valujskikh, J. Liu, N. Muqim, Y. Xu, and M.E. Medof. 2005. Decay-accelerating factor modulates induction of T cell immunity. *J Exp Med* 201:1523-1530.

Herve, M.A., H. Buteau-Lozano, S. Mourah, F. Calvo, and M. Perrot-Applanat. 2005. VEGF189 stimulates endothelial cells proliferation and migration in vitro and up-regulates the expression of Flk-1/KDR mRNA. *Exp Cell Res* 309:24-31.

Herzenberg, L.A. 2000. B-1 cells: the lineage question revisited. *Immunol Rev* 175:9-22.

Herzog, S., M. Reth, and H. Jumaa. 2009. Regulation of B-cell proliferation and differentiation by pre-B-cell receptor signalling. *Nat Rev Immunol* 9:195-205.

Hikida, M., M. Mori, T. Takai, K. Tomochika, K. Hamatani, and H. Ohmori. 1996. Reexpression of RAG-1 and RAG-2 genes in activated mature mouse B cells. *Science* 274:2092-2094.

Hildemann, W.H., and R.L. Walford. 1960. Chronic skin homograft rejection in the Syrian hamster. *Ann N Y Acad Sci* 87:56-77.

Hillebrands, J.L., F.A. Klatter, B.M. van den Hurk, E.R. Popa, P. Nieuwenhuis, and J. Rozing. 2001. Origin of neointimal endothelium and alpha-actin-positive smooth muscle cells in transplant arteriosclerosis. *J Clin Invest* 107:1411-1422.

Ho, E.K., G. Vlad, E.R. Vasilescu, L. de la Torre, A.I. Colovai, E. Burke, M. Deng, J. Schwartz, C. Marboe, D. Mancini, G. Opelz, and N. Suciu-Foca. 2011. Pre- and posttransplantation allosensitization in heart allograft recipients: major impact of de novo alloantibody production on allograft survival. *Hum Immunol* 72:5-10.

Holmen, C., E. Elsheikh, M. Christensson, J. Liu, A.S. Johansson, A.R. Qureshi, S. Jalkanen, and S. Sumitran-Holgersson. 2007. Anti endothelial cell autoantibodies selectively activate SAPK/JNK signalling in Wegener's granulomatosis. *J Am Soc Nephrol* 18:2497-2508.

Honjo, K., X. Yan Xu, J.A. Kapp, and R.P. Bucy. 2004. Evidence for cooperativity in the rejection of cardiac grafts mediated by CD4 TCR Tg T cells specific for a defined allopeptide. *Am J Transplant* 4:1762-1768.

Hori, S., S. Kitagawa, H. Iwata, T. Ochiai, K. Isono, T. Hamaoka, and H. Fujiwara. 1992. Cell-cell interaction in graft rejection responses: induction of anti-allo-class I H-2 tolerance is prevented by immune responses against allo-class II H-2 antigens coexpressed on tolerogen. *J Exp Med* 175:99-109.

Hosenpud, J.D., G.D. Shipley, and C.R. Wagner. 1992. Cardiac allograft vasculopathy: current concepts, recent developments, and future directions. *J Heart Lung Transplant* 11:9-23.

Hoyer, B.F., R.A. Manz, A. Radbruch, and F. Hiepe. 2005. Long-lived plasma cells and their contribution to autoimmunity. *Ann N Y Acad Sci* 1050:124-133.

Hoyer, B.F., K. Moser, A.E. Hauser, A. Peddinghaus, C. Voigt, D. Eilat, A. Radbruch, F. Hiepe, and R.A. Manz. 2004. Short-lived plasmablasts and long-lived plasma cells contribute to chronic humoral autoimmunity in NZB/W mice. *J Exp Med* 199:1577-1584.

Huygen, K., A. Drowart, M. Harboe, R. ten Berg, J. Cogniaux, and J.P. Van Vooren. 1993. Influence of genes from the major histocompatibility complex on the antibody repertoire against culture filtrate antigens in mice infected with live Mycobacterium bovis BCG. *Infect Immun* 61:2687-2693.

Ichikawa, T., E. Nakayama, A. Uenaka, M. Monden, and T. Mori. 1987. Effector cells in allelic H-2 class I-incompatible skin graft rejection. *J Exp Med* 166:982-990.

Ishii, D., J.M. Rosenblum, T. Nozaki, A.D. Schenk, K. Setoguchi, C.A. Su, V. Gorbacheva, W.M. Baldwin, 3rd, A. Valujskikh, and R.L. Fairchild. 2014. Novel CD8 T cell alloreactivities in CCR5-deficient recipients of class II MHC disparate kidney grafts. *J Immunol* 193:3816-3824.

Jacob, J., G. Kelsoe, K. Rajewsky, and U. Weiss. 1991. Intraclonal generation of antibody mutants in germinal centres. *Nature* 354:389-392.

Jin, Y.P., P.T. Jindra, K.W. Gong, E.J. Lepin, and E.F. Reed. 2005. Anti-HLA class I antibodies activate endothelial cells and promote chronic rejection. *Transplantation* 79:S19-21.

Jin, Y.P., Y. Korin, X. Zhang, P.T. Jindra, E. Rozengurt, and E.F. Reed. 2007. RNA interference elucidates the role of focal adhesion kinase in HLA class I-mediated focal adhesion complex formation and proliferation in human endothelial cells. *J Immunol* 178:7911-7922.

Jin, Y.P., N.M. Valenzuela, M.E. Ziegler, E. Rozengurt, and E.F. Reed. 2014. Everolimus inhibits anti-HLA I antibody-mediated endothelial cell signaling, migration and proliferation more potently than sirolimus. *Am J Transplant* 14:806-819.

Jindra, P.T., A. Hsueh, L. Hong, D. Gjertson, X.D. Shen, F. Gao, J. Dang, P.S. Mischel, W.M. Baldwin, 3rd, M.C. Fishbein, J.W. Kupiec-Weglinski, and E.F. Reed. 2008. Anti-MHC class I antibody activation of proliferation and survival signaling in murine cardiac allografts. *J Immunol* 180:2214-2224.

Johnston, R.J., A.C. Poholek, D. DiToro, I. Yusuf, D. Eto, B. Barnett, A.L. Dent, J. Craft, and S. Crotty. 2009. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* 325:1006-1010.

Jurcevic, S., M.E. Ainsworth, A. Pomerance, J.D. Smith, D.R. Robinson, M.J. Dunn, M.H. Yacoub, and M.L. Rose. 2001. Antivimentin antibodies are an independent predictor of transplant-associated coronary artery disease after cardiac transplantation. *Transplantation* 71:886-892.

Kalache, S., R. Dinavahi, S. Pinney, A. Mehrotra, M.W. Cunningham, and P.S. Heeger. 2011. Anticardiac myosin immunity and chronic allograft vasculopathy in heart transplant recipients. *J Immunol* 187:1023-1030.

Kamperschroer, C., J.P. Dibble, D.L. Meents, P.L. Schwartzberg, and S.L. Swain. 2006. SAP is required for Th cell function and for immunity to influenza. *J Immunol* 177:5317-5327.

Kelsoe, G. 1996. The germinal center: a crucible for lymphocyte selection. *Semin Immunol* 8:179-184.

Kim, E.J., J. Kwun, A.C. Gibby, J.J. Hong, A.B. Farris, 3rd, N.N. Iwakoshi, F. Villinger, A.D. Kirk, and S.J. Knechtle. 2014. Costimulation blockade alters germinal center responses and prevents antibody-mediated rejection. *Am J Transplant* 14:59-69.

King, C., S.G. Tangye, and C.R. Mackay. 2008. T follicular helper (TFH) cells in normal and dysregulated immune responses. *Annu Rev Immunol* 26:741-766.

Kitagawa, S., S. Sato, S. Hori, T. Hamaoka, and H. Fujiwara. 1990. Induction of anti-allo-class I H-2 tolerance by inactivation of CD8+ helper T cells, and reversal of tolerance through introduction of third-party helper T cells. *J Exp Med* 172:105-113.

Klein, L., M. Hinterberger, G. Wirnsberger, and B. Kyewski. 2009. Antigen presentation in the thymus for positive selection and central tolerance induction. *Nat Rev Immunol* 9:833-844.

Koh, K.P., Y. Wang, T. Yi, S.L. Shiao, M.I. Lorber, W.C. Sessa, G. Tellides, and J.S. Pober. 2004. T cell-mediated vascular dysfunction of human allografts results from IFN-gamma dysregulation of NO synthase. *J Clin Invest* 114:846-856.

Kroemer, A., X. Xiao, N. Degauque, K. Edtinger, H. Wei, G. Demirci, and X.C. Li. 2008. The innate NK cells, allograft rejection, and a key role for IL-15. *J Immunol* 180:7818-7826.

Labarrere, C.A., D. Pitts, D.R. Nelson, and W.P. Faulk. 1995. Vascular tissue plasminogen activator and the development of coronary artery disease in heart-transplant recipients. *N Engl J Med* 333:1111-1116.

Laszlo, G., K.S. Hathcock, H.B. Dickler, and R.J. Hodes. 1993. Characterization of a novel cell-surface molecule expressed on subpopulations of activated T and B cells. *J Immunol* 150:5252-5262.

Lawson, C., A.L. Holder, R.E. Stanford, J. Smith, and M.L. Rose. 2005. Anti-intercellular adhesion molecule-1 antibodies in sera of heart transplant recipients: a role in endothelial cell activation. *Transplantation* 80:264-271.

Leadbetter, E.A., I.R. Rifkin, A.M. Hohlbaum, B.C. Beaudette, M.J. Shlomchik, and A. Marshak-Rothstein. 2002. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature* 416:603-607.

Lee, P.C., P.I. Terasaki, S.K. Takemoto, P.H. Lee, C.J. Hung, Y.L. Chen, A. Tsai, and H.Y. Lei. 2002. All chronic rejection failures of kidney transplants were preceded by the development of HLA antibodies. *Transplantation* 74:1192-1194.

Lei, Y.M., L. Chen, Y. Wang, A.T. Stefka, L.L. Molinero, B. Theriault, K. Aquino-Michaels, A.S. Sivan, C.R. Nagler, T.F. Gajewski, A.S. Chong, C. Bartman, and M.L. Alegre. 2016. The composition of the microbiota modulates allograft rejection. *J Clin Invest* 126:2736-2744.

Leong, H.S., B.M. Mahesh, J.R. Day, J.D. Smith, A.D. McCormack, G. Ghimire, T.J. Podor, and M.L. Rose. 2008. Vimentin autoantibodies induce platelet activation and formation of platelet-leukocyte conjugates via platelet-activating factor. *J Leukoc Biol* 83:263-271.

Li, C., and C.W. Yang. 2009. The pathogenesis and treatment of chronic allograft nephropathy. *Nat Rev Nephrol* 5:513-519.

Li, H., Y. Jiang, E.L. Prak, M. Radic, and M. Weigert. 2001. Editors and editing of anti-DNA receptors. *Immunity* 15:947-957.

Li, H.W., and M. Sykes. 2012. Emerging concepts in haematopoietic cell transplantation. *Nat Rev Immunol* 12:403-416.

Libby, P., and J.S. Pober. 2001. Chronic rejection. *Immunity* 14:387-397.

Lin, H., J.E. Wilson, T.J. Kendall, S.J. Radio, F.J. Cornhill, E. Herderick, G.L. Winters, M.R. Costanzo, T. Porter, S.L. Thieszen, and et al. 1994. Comparable proximal and distal severity of intimal thickening and size of epicardial coronary arteries in transplant arteriopathy of human cardiac allografts. *J Heart Lung Transplant* 13:824-833.

Linterman, M.A., L. Beaton, D. Yu, R.R. Ramiscal, M. Srivastava, J.J. Hogan, N.K. Verma, M.J. Smyth, R.J. Rigby, and C.G. Vinuesa. 2010. IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. *J Exp Med* 207:353-363.

Linterman, M.A., A. Liston, and C.G. Vinuesa. 2012. T-follicular helper cell differentiation and the co-option of this pathway by non-helper cells. *Immunol Rev* 247:143-159.

Linterman, M.A., R.J. Rigby, R.K. Wong, D. Yu, R. Brink, J.L. Cannons, P.L. Schwartzberg, M.C. Cook, G.D. Walters, and C.G. Vinuesa. 2009. Follicular helper T cells are required for systemic autoimmunity. *J Exp Med* 206:561-576.

Liu, S., M.G. Velez, J. Humann, S. Rowland, F.J. Conrad, R. Halverson, R.M. Torres, and R. Pelanda. 2005. Receptor editing can lead to allelic inclusion and development of B cells that retain antibodies reacting with high avidity autoantigens. *J Immunol* 175:5067-5076.

Liu, Y.J., J. Zhang, P.J. Lane, E.Y. Chan, and I.C. MacLennan. 1991. Sites of specific B cell activation in primary and secondary responses to T cell-dependent and T cell-independent antigens. *Eur J Immunol* 21:2951-2962.

Liu, Z., Y.K. Sun, Y.P. Xi, A. Maffei, E. Reed, P. Harris, and N. Suciu-Foca. 1993. Contribution of direct and indirect recognition pathways to T cell alloreactivity. *J Exp Med* 177:1643-1650.

Louis, S., C. Braudeau, M. Giral, A. Dupont, F. Moizant, N. Robillard, A. Moreau, J.P. Soulillou, and S. Brouard. 2006. Contrasting CD25hiCD4+T cells/FOXP3 patterns in chronic rejection and operational drug-free tolerance. *Transplantation* 81:398-407.

Lovegrove, E., G.J. Pettigrew, E.M. Bolton, and J.A. Bradley. 2001. Epitope mapping of the indirect T cell response to allogeneic class I mhc: sequences shared by donor and recipient MHC may prime T cells that provide help for alloantibody production. *J Immunol* 167:4338-4344.

Luning Prak, E.T., M. Monestier, and R.A. Eisenberg. 2011. B cell receptor editing in tolerance and autoimmunity. *Ann N Y Acad Sci* 1217:96-121.

Luo, B., W.F. Chan, A.M. Shapiro, and C.C. Anderson. 2007. Non-myeloablative mixed chimerism approaches and tolerance, a split decision. *Eur J Immunol* 37:1233-1242.

Lustgraaf, E.C., R.B. Fuson, and E.J. Eichwald. 1960. *Transpl. Bull.* 26:

Luther, S.A., I. Maillard, F. Luthi, L. Scarpellino, H. Diggelmann, and H. Acha-Orbea. 1997. Early neutralizing antibody response against mouse mammary tumor virus: critical role of viral infection and superantigen-reactive T cells. *J Immunol* 159:2807-2814.

Luzina, I.G., S.P. Atamas, C.E. Storrer, L.C. daSilva, G. Kelsoe, J.C. Papadimitriou, and B.S. Handwerger. 2001. Spontaneous formation of germinal centers in autoimmune mice. *J Leukoc Biol* 70:578-584.

Macdonald, W.A., Z. Chen, S. Gras, J.K. Archbold, F.E. Tynan, C.S. Clements, M. Bharadwaj, L. Kjer-Nielsen, P.M. Saunders, M.C. Wilce, F. Crawford, B. Stadinsky, D. Jackson, A.G. Brooks, A.W. Purcell, J.W. Kappler, S.R. Burrows, J. Rossjohn, and J. McCluskey. 2009. T cell allorecognition via molecular mimicry. *Immunity* 31:897-908.

MacLennan, I.C. 1994. Germinal centers. *Annu Rev Immunol* 12:117-139.

MacLennan, I.C., K.M. Toellner, A.F. Cunningham, K. Serre, D.M. Sze, E. Zuniga, M.C. Cook, and C.G. Vinuesa. 2003. Extrafollicular antibody responses. *Immunol Rev* 194:8-18.

Mahesh, B., H.S. Leong, A. McCormack, P. Sarathchandra, A. Holder, and M.L. Rose. 2007. Autoantibodies to vimentin cause accelerated rejection of cardiac allografts. *Am J Pathol* 170:1415-1427.

Mamula, M.J. 1998. Epitope spreading: the role of self peptides and autoantigen processing by B lymphocytes. *Immunol Rev* 164:231-239.

Mamula, M.J., and C.A. Janeway, Jr. 1993. Do B cells drive the diversification of immune responses? *Immunol Today* 14:151-152; discussion 153-154.

Mamula, M.J., R.H. Lin, C.A. Janeway, Jr., and J.A. Hardin. 1992. Breaking T cell tolerance with foreign and self co-immunogens. A study of autoimmune B and T cell epitopes of cytochrome c. *J Immunol* 149:789-795.

Manoury, B., D. Mazzeo, L. Fugger, N. Viner, M. Ponsford, H. Streeter, G. Mazza, D.C. Wraith, and C. Watts. 2002. Destructive processing by asparagine endopeptidase limits presentation of a dominant T cell epitope in MBP. *Nat Immunol* 3:169-174.

Manz, R.A., S. Arce, G. Cassese, A.E. Hauser, F. Hiepe, and A. Radbruch. 2002. Humoral immunity and long-lived plasma cells. *Curr Opin Immunol* 14:517-521.

Manz, R.A., A. Thiel, and A. Radbruch. 1997. Lifetime of plasma cells in the bone marrow. *Nature* 388:133-134.

Marino, J., J. Paster, and G. Benichou. 2016. Allorecognition by T Lymphocytes and Allograft Rejection. *Front Immunol* 7:582.

Marrack, P., J. Kappler, and B.L. Kotzin. 2001. Autoimmune disease: why and where it occurs. *Nat Med* 7:899-905.

Martin, F., and J.F. Kearney. 2000. B-cell subsets and the mature preimmune repertoire. Marginal zone and B1 B cells as part of a "natural immune memory". *Immunol Rev* 175:70-79.

Martin, F., and J.F. Kearney. 2002. Marginal-zone B cells. *Nat Rev Immunol* 2:323-335.

Martinez-Llordella, M., I. Puig-Pey, G. Orlando, M. Ramoni, G. Tisone, A. Rimola, J. Lerut, D. Latinne, C. Margarit, I. Bilbao, S. Brouard, M. Hernandez-Fuentes, J.P. Soulillou, and A. Sanchez-Fueyo. 2007. Multiparameter immune profiling of operational tolerance in liver transplantation. *Am J Transplant* 7:309-319.

Mathes, D.W., M.A. Randolph, M.G. Solari, J.A. Nazzal, G.P. Nielsen, J.S. Arn, D.H. Sachs, and W.P. Lee. 2003. Split tolerance to a composite tissue allograft in a swine model. *Transplantation* 75:25-31.

Mauiyyedi, S., and R.B. Colvin. 2002. Humoral rejection in kidney transplantation: new concepts in diagnosis and treatment. *Curr Opin Nephrol Hypertens* 11:609-618.

McCausland, M.M., I. Yusuf, H. Tran, N. Ono, Y. Yanagi, and S. Crotty. 2007. SAP regulation of follicular helper CD4 T cell development and humoral immunity is independent of SLAM and Fyn kinase. *J Immunol* 178:817-828.

McIntyre, K.R., and J.G. Seidman. 1984. Nucleotide sequence of mutant I-A beta bm12 gene is evidence for genetic exchange between mouse immune response genes. *Nature* 308:551-553.

McKenzie, I.F., G.M. Morgan, M.S. Sandrin, M.M. Michaelides, R.W. Melvold, and H.I. Kohn. 1979. B6.C-H-2bm12. A new H-2 mutation in the I region in the mouse. *J Exp Med* 150:1323-1338.

Medawar, P.B. 1944. The behaviour and fate of skin autografts and skin homografts in rabbits: A report to the War Wounds Committee of the Medical Research Council. *J Anat* 78:176-199.

Mengle-Gaw, L., S. Conner, H.O. McDevitt, and C.G. Fathman. 1984. Gene conversion between murine class II major histocompatibility complex loci. Functional and molecular evidence from the bm 12 mutant. *J Exp Med* 160:1184-1194.

Methe, H., S. Brunner, D. Wiegand, M. Nabauer, J. Koglin, and E.R. Edelman. 2005a. Enhanced T-helper-1 lymphocyte activation patterns in acute coronary syndromes. *J Am Coll Cardiol* 45:1939-1945.

Methe, H., D. Wiegand, U. Welsch, F. Krombach, B. Meiser, M. Nabauer, and J. Koglin. 2005b. Peripheral expansion of circulating T-helper 1 cells predicts coronary endothelial dysfunction after cardiac transplantation. *J Heart Lung Transplant* 24:833-840.

Mitchell, R.N., and P. Libby. 2007. Vascular remodeling in transplant vasculopathy. *Circ Res* 100:967-978.

Mitchison, N.A. 1971. The carrier effect in the secondary response to hapten-protein conjugates. II. Cellular cooperation. *Eur J Immunol* 1:18-27.

Miyazaki, T., P. Wolf, S. Tourne, C. Waltzinger, A. Dierich, N. Barois, H. Ploegh, C. Benoist, and D. Mathis. 1996. Mice lacking H2-M complexes, enigmatic elements of the MHC class II peptide-loading pathway. *Cell* 84:531-541.

Morris, S.C., R.L. Cheek, P.L. Cohen, and R.A. Eisenberg. 1990a. Allotype-specific immunoregulation of autoantibody production by host B cells in chronic graft-versus host disease. *J Immunol* 144:916-922.

Morris, S.C., R.L. Cheek, P.L. Cohen, and R.A. Eisenberg. 1990b. Autoantibodies in chronic graft versus host result from cognate T-B interactions. *J Exp Med* 171:503-517.

Motallebzadeh, R., S. Rehakova, T.M. Conlon, T.S. Win, C.J. Callaghan, M. Goddard, E.M. Bolton, N.H. Ruddle, J.A. Bradley, and G.J. Pettigrew. 2012. Blocking lymphotoxin signaling abrogates the development of ectopic lymphoid tissue within cardiac allografts and inhibits effector antibody responses. *Faseb J* 26:51-62.

Muramatsu, M., K. Kinoshita, S. Fagarasan, S. Yamada, Y. Shinkai, and T. Honjo. 2000. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* 102:553-563.

Muramatsu, M., V.S. Sankaranand, S. Anant, M. Sugai, K. Kinoshita, N.O. Davidson, and T. Honjo. 1999. Specific expression of activation-induced cytidine deaminase (AID), a novel member of the RNA-editing deaminase family in germinal center B cells. *J Biol Chem* 274:18470-18476.

Murasawa, M., S. Okada, S. Obata, M. Hatano, H. Moriya, and T. Tokuhisa. 2002. GL7 defines the cycling stage of pre-B cells in murine bone marrow. *Eur J Immunol* 32:291-298.

Nadarajah, L., N. Ashman, R. Thuraisingham, C. Barber, S. Allard, and L. Green. 2013. Literature review of passenger lymphocyte syndrome following renal transplantation and two case reports. *Am J Transplant* 13:1594-1600.

Nafady-Hego, H., Y. Li, H. Ohe, X. Zhao, N. Satoda, S. Sakaguchi, K. Wood, S. Uemoto, and T. Koshiba. 2010. The generation of donor-specific CD4+CD25++CD45RA+ naive regulatory T cells in operationally tolerant patients after pediatric living-donor liver transplantation. *Transplantation* 90:1547-1555.

Nagano, H., P. Libby, M.K. Taylor, S. Hasegawa, J.L. Stinn, G. Becker, N.L. Tilney, and R.N. Mitchell. 1998. Coronary arteriosclerosis after T-cell-mediated injury in transplanted mouse hearts: role of interferon-gamma. *Am J Pathol* 152:1187-1197.

Nagano, H., R.N. Mitchell, M.K. Taylor, S. Hasegawa, N.L. Tilney, and P. Libby. 1997. Interferon-gamma deficiency prevents coronary arteriosclerosis but not myocardial rejection in transplanted mouse hearts. *J Clin Invest* 100:550-557.

Nakashima, S., Z. Qian, S. Rahimi, B.A. Wasowska, and W.M. Baldwin, 3rd. 2002. Membrane attack complex contributes to destruction of vascular integrity in acute lung allograft rejection. *J Immunol* 169:4620-4627.

Nikolic, B., T. Onoe, Y. Takeuchi, Z. Khalpey, V. Primo, I. Leykin, R.N. Smith, and M. Sykes. 2010. Distinct requirements for achievement of allotolerance versus reversal of autoimmunity via nonmyeloablative mixed chimerism induction in NOD mice. *Transplantation* 89:23-32.

Nurieva, R.I., Y. Chung, D. Hwang, X.O. Yang, H.S. Kang, L. Ma, Y.H. Wang, S.S. Watowich, A.M. Jetten, Q. Tian, and C. Dong. 2008. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity* 29:138-149.

Nurieva, R.I., Y. Chung, G.J. Martinez, X.O. Yang, S. Tanaka, T.D. Matskevitch, Y.H. Wang, and C. Dong. 2009. Bcl6 mediates the development of T follicular helper cells. *Science* 325:1001-1005.

Oberbarnscheidt, M.H., Q. Zeng, Q. Li, H. Dai, A.L. Williams, W.D. Shlomchik, D.M. Rothstein, and F.G. Lakkis. 2014. Non-self recognition by monocytes initiates allograft rejection. *J Clin Invest* 124:3579-3589.

Oliver, A.M., F. Martin, and J.F. Kearney. 1999. IgMhighCD21high lymphocytes enriched in the splenic marginal zone generate effector cells more rapidly than the bulk of follicular B cells. *J Immunol* 162:7198-7207.

Pasare, C., and R. Medzhitov. 2005. Control of B-cell responses by Toll-like receptors. *Nature* 438:364-368.

Peng, Q., K. Li, K. Anderson, C.A. Farrar, B. Lu, R.A. Smith, S.H. Sacks, and W. Zhou. 2008. Local production and activation of complement up-regulates the allostimulatory function of dendritic cells through C3a-C3aR interaction. *Blood* 111:2452-2461.

Piotti, G., A. Palmisano, U. Maggiore, and C. Buzio. 2014. Vascular endothelium as a target of immune response in renal transplant rejection. *Front Immunol* 5:505.

Pratschke, J., P. Neuhaus, and S.G. Tullius. 2005. What can be learned from brain-death models? *Transpl Int* 18:15-21.

Qi, H. 2012. From SAP-less T cells to helpless B cells and back: dynamic T-B cell interactions underlie germinal center development and function. *Immunol Rev* 247:24-35.

Qi, H., J.L. Cannons, F. Klauschen, P.L. Schwartzberg, and R.N. Germain. 2008. SAP-controlled T-B cell interactions underlie germinal centre formation. *Nature* 455:764-769.

Qian, S., L. Lu, Y. Li, F. Fu, W. Li, T.E. Starzl, A.W. Thomson, and J.J. Fung. 1997. Apoptosis of graft-infiltrating cytotoxic T cells: a mechanism underlying "split tolerance" in mouse liver transplantation. *Transplant Proc* 29:1168-1169.

Qureshi, M.S., J. Alsughayyir, M. Chhabra, J.M. Ali, J.A. Bradley, E.M. Bolton, M.J. Goddard, C. Devine, T.M. Conlon, M.A. Linterman, R. Motallebzadeh, and G.J. Pettigrew. 2019. Data regarding Transplant Induced Germinal Center Humoral Autoimmunity. *Data in Brief Submitted*:

Qureshi, M.S., J. Alsughayyir, M. Chhabra, J.M. Ali, M.J. Goddard, C. Devine, T.M. Conlon, M.A. Linterman, R. Motallebzadeh, and G.J. Pettigrew. 2018. Germinal center humoral autoimmunity mediates progression of allograft vasculopathy independently from recipient alloimmunity. *J Autoimmun In press*

Raedler, H., M. Yang, P.N. Lalli, M.E. Medof, and P.S. Heeger. 2009. Primed CD8(+) T-cell responses to allogeneic endothelial cells are controlled by local complement activation. *Am J Transplant* 9:1784-1795.

Rahmani, M., R.P. Cruz, D.J. Granville, and B.M. McManus. 2006. Allograft vasculopathy versus atherosclerosis. *Circ Res* 99:801-815.

Rajewsky, K., V. Schirrmacher, S. Nase, and N.K. Jerne. 1969. The requirement of more than one antigenic determinant for immunogenicity. *J Exp Med* 129:1131-1143.

Rasheed, Z., M.W. Khan, and R. Ali. 2006. Hydroxyl radical modification of human serum albumin generated cross reactive antibodies. *Autoimmunity* 39:479-488.

Regele, H., G.A. Bohmig, A. Habicht, D. Gollowitzer, M. Schillinger, S. Rockenschaub, B. Watschinger, D. Kerjaschki, and M. Exner. 2002. Capillary deposition of complement split product C4d in renal allografts is associated with basement membrane injury in peritubular and glomerular capillaries: a contribution of humoral immunity to chronic allograft rejection. *J Am Soc Nephrol* 13:2371-2380.

Rodriguez, E.R., D.V. Skojec, C.D. Tan, A.A. Zachary, E.K. Kasper, J.V. Conte, and W.M. Baldwin, 3rd. 2005. Antibody-mediated rejection in human cardiac allografts: evaluation of immunoglobulins and complement activation products C4d and C3d as markers. *Am J Transplant* 5:2778-2785.

Roedder, S., L. Li, M.N. Alonso, S.C. Hsieh, M.T. Vu, H. Dai, T.K. Sigdel, I. Bostock, C. Macedo, D. Metes, A. Zeevi, R. Shapiro, O. Salvatierra, J. Scandling, J. Alberu, E. Engleman, and M.M. Sarwal. 2015. A Three-Gene Assay for Monitoring Immune Quiescence in Kidney Transplantation. *J Am Soc Nephrol* 26:2042-2053.

Rogers, N.J., and R.I. Lechler. 2001. Allorecognition. *Am J Transplant* 1:97-102.

Rolink, A.G., H. Gleichmann, and E. Gleichmann. 1983. Diseases caused by reactions of T lymphocytes to incompatible structures of the major histocompatibility complex. VII. Immune-complex glomerulonephritis. *J Immunol* 130:209-215.

Romano, M., G. Fanelli, C.J. Albany, G. Giganti, and G. Lombardi. 2019. Past, Present, and Future of Regulatory T Cell Therapy in Transplantation and Autoimmunity. *Front Immunol* 10:43.

Rose, M.L. 2013. Role of anti-vimentin antibodies in allograft rejection. *Hum Immunol* 74:1459-1462.

Rose, M.L., M.S. Birbeck, V.J. Wallis, J.A. Forrester, and A.J. Davies. 1980. Peanut lectin binding properties of germinal centres of mouse lymphoid tissue. *Nature* 284:364-366.

Rose, M.L., and J.D. Smith. 2009. Clinical relevance of complement-fixing antibodies in cardiac transplantation. *Hum Immunol* 70:605-609.

Rosenberg, A.S., T. Mizuochi, S.O. Sharrow, and A. Singer. 1987. Phenotype, specificity, and function of T cell subsets and T cell interactions involved in skin allograft rejection. *J Exp Med* 165:1296-1315.

Rosenberg, A.S., T.I. Munitz, T.G. Maniero, and A. Singer. 1991. Cellular basis of skin allograft rejection across a class I major histocompatibility barrier in mice depleted of CD8+ T cells in vivo. *J Exp Med* 173:1463-1471.

Rothenberg, E.V., J.E. Moore, and M.A. Yui. 2008. Launching the T-cell-lineage developmental programme. *Nat Rev Immunol* 8:9-21.

Ruggiero, R., J. Muz, R. Fietsam, Jr., G.A. Thomas, R.J. Welsh, J.E. Miller, L.W. Stephenson, and F.A. Baciewicz, Jr. 1993. Reestablishment of lymphatic drainage after canine lung transplantation. *J Thorac Cardiovasc Surg* 106:167-171.

Russell, M.E. 2000. Cardiac allograft vasculopathy--a changing perspective. *Z Kardiol* 89 Suppl 9:IX/6-10.

Saadi, S., R.A. Holzknecht, C.P. Patte, and J.L. Platt. 2000. Endothelial cell activation by pore-forming structures: pivotal role for interleukin-1alpha. *Circulation* 101:1867-1873.

Saiki, T., T. Ezaki, M. Ogawa, and K. Matsuno. 2001. Trafficking of host- and donor-derived dendritic cells in rat cardiac transplantation: allosensitization in the spleen and hepatic nodes. *Transplantation* 71:1806-1815.

Saint-Ruf, C., K. Ungewiss, M. Groettrup, L. Bruno, H.J. Fehling, and H. von Boehmer. 1994. Analysis and expression of a cloned pre-T cell receptor gene. *Science* 266:1208-1212.

Sarwal, M.M. 2016. Fingerprints of transplant tolerance suggest opportunities for immunosuppression minimization. *Clin Biochem* 49:404-410.

Sayegh, M.H., Z. Wu, W.W. Hancock, P.B. Langmuir, M. Mata, S. Sandner, K. Kishimoto, M. Sho, E. Palmer, R.N. Mitchell, and L.A. Turka. 2003. Allograft rejection in a new allospecific CD4+ TCR transgenic mouse. *Am J Transplant* 3:381-389.

Schaerli, P., K. Willimann, A.B. Lang, M. Lipp, P. Loetscher, and B. Moser. 2000. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J Exp Med* 192:1553-1562.

Schmitt, E.G., and C.B. Williams. 2013. Generation and function of induced regulatory T cells. *Front Immunol* 4:152.

Schmitt, N., R. Morita, L. Bourdery, S.E. Bentebibel, S.M. Zurawski, J. Banchereau, and H. Ueno. 2009. Human dendritic cells induce the differentiation of interleukin-21-producing T follicular helper-like cells through interleukin-12. *Immunity* 31:158-169.

Schnickel, G.T., D. Whiting, G.R. Hsieh, J.J. Yun, M.P. Fischbein, M.C. Fishbein, W. Yao, A. Shfizadeh, and A. Ardehali. 2004. CD8 lymphocytes are sufficient for the development of chronic rejection. *Transplantation* 78:1634-1639.

Schwickert, T.A., B. Alabyev, T. Manser, and M.C. Nussenzweig. 2009. vGerminal center reutilization by newly activated B cells. *J Exp Med* 206:2907-2914.

Sekiguchi, D.R., R.A. Eisenberg, and M. Weigert. 2003. Secondary heavy chain rearrangement: a mechanism for generating anti-double-stranded DNA B cells. *J Exp Med* 197:27-39.

Sekiguchi, D.R., S.M. Jainandunsing, M.L. Fields, M.A. Maldonado, M.P. Madaio, J. Erikson, M. Weigert, and R.A. Eisenberg. 2002. Chronic graft-versus-host in Ig knockin transgenic mice abrogates B cell tolerance in anti-double-stranded DNA B cells. *J Immunol* 168:4142-4153.

Sewell, A.K. 2012. Why must T cells be cross-reactive? *Nature Reviews Immunology* 12:669.

Shapiro-Shelef, M., K.I. Lin, L.J. McHeyzer-Williams, J. Liao, M.G. McHeyzer-Williams, and K. Calame. 2003. Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. *Immunity* 19:607-620.

Sharples, L.D., C.H. Jackson, J. Parameshwar, J. Wallwork, and S.R. Large. 2003. Diagnostic accuracy of coronary angiography and risk factors for post-heart-transplant cardiac allograft vasculopathy. *Transplantation* 76:679-682.

Shi, C., W.S. Lee, Q. He, D. Zhang, D.L. Fletcher, Jr., J.B. Newell, and E. Haber. 1996. Immunologic basis of transplant-associated arteriosclerosis. *Proc Natl Acad Sci U S A* 93:4051-4056.

Shinoda, K., T. Akiyoshi, C.M. Chase, E.A. Farkash, D.K. Ndishabandi, C.M. Raczek, D.P. Sebastian, P. Della Pelle, P.S. Russell, J.C. Madsen, R.B. Colvin, and A. Alessandrini. 2014. Depletion of foxp3(+) T cells abrogates tolerance of skin and heart allografts in murine mixed chimeras without the loss of mixed chimerism. *Am J Transplant* 14:2263-2274.

Shlomchik, M.J., J.E. Craft, and M.J. Mamula. 2001. From T to B and back again: positive feedback in systemic autoimmune disease. *Nat Rev Immunol* 1:147-153.

Shulman, Z., A.D. Gitlin, S. Targ, M. Jankovic, G. Pasqual, M.C. Nussenzweig, and G.D. Victora. 2013. T follicular helper cell dynamics in germinal centers. *Science* 341:673-677.

Singh, R.R., and B.H. Hahn. 1998. Reciprocal T-B determinant spreading develops spontaneously in murine lupus: implications for pathogenesis. *Immunol Rev* 164:201-208.

Slifka, M.K., R. Antia, J.K. Whitmire, and R. Ahmed. 1998. Humoral immunity due to long-lived plasma cells. *Immunity* 8:363-372.

Slifka, M.K., M. Matloubian, and R. Ahmed. 1995. Bone marrow is a major site of long-term antibody production after acute viral infection. *J Virol* 69:1895-1902.

Smith, K.G., T.D. Hewitson, G.J. Nossal, and D.M. Tarlinton. 1996. The phenotype and fate of the antibody-forming cells of the splenic foci. *Eur J Immunol* 26:444-448.

Smith, R.N., N. Brousaides, L. Grazette, S. Saidman, M. Semigran, T. Disalvo, J. Madsen, G.W. Dec, A.R. Perez-Atayde, and A.B. Collins. 2005. C4d deposition in cardiac allografts correlates with alloantibody. *J Heart Lung Transplant* 24:1202-1210.

Smyth, L.A., O.B. Herrera, D. Golshayan, G. Lombardi, and R.I. Lechler. 2006. A novel pathway of antigen presentation by dendritic and endothelial cells: Implications for allorecognition and infectious diseases. *Transplantation* 82:S15-18.

Snell, G.D. 1948. Methods for the study of histocompatibility genes. *J Genet* 49:87-108.

Stary, H.C. 1989. Evolution and progression of atherosclerotic lesions in coronary arteries of children and young adults. *Arteriosclerosis* 9:I19-32.

Steele, D.J., T.M. Laufer, S.T. Smiley, Y. Ando, M.J. Grusby, L.H. Glimcher, and H. Auchincloss, Jr. 1996. Two levels of help for B cell alloantibody production. *J Exp Med* 183:699-703.

Stegall, M.D. 2010. Evidence that alloantibody is a major risk factor for graft loss and death in living-donor liver transplantation. *Surgery* 147:845-846.

Stockinger, B., T. Zal, A. Zal, and D. Gray. 1996. B cells solicit their own help from T cells [see comments]. *J Exp Med* 183:891-899.

Strainic, M.G., J. Liu, D. Huang, F. An, P.N. Lalli, N. Muqim, V.S. Shapiro, G.R. Dubyak, P.S. Heeger, and M.E. Medof. 2008. Locally produced complement fragments C5a and C3a provide both costimulatory and survival signals to naive CD4+ T cells. *Immunity* 28:425-435.

Stuart, P.M., B. Beck-Maier, and R.W. Melvold. 1984. Provocation of skin graft rejection across murine class II differences by non--bone-marrow-derived cells. *Transplantation* 37:393-396.

Suchin, E.J., P.B. Langmuir, E. Palmer, M.H. Sayegh, A.D. Wells, and L.A. Turka. 2001. Quantifying the frequency of alloreactive T cells in vivo: new answers to an old question. *J Immunol* 166:973-981.

Suciu-Foca, N., P.E. Harris, and R. Cortesini. 1998. Intramolecular and intermolecular spreading during the course of organ allograft rejection. *Immunol Rev* 164:241-246.

Sze, D.M., K.M. Toellner, C. Garcia de Vinuesa, D.R. Taylor, and I.C. MacLennan. 2000. Intrinsic constraint on plasmablast growth and extrinsic limits of plasma cell survival. *J Exp Med* 192:813-821.

Takahashi, Y., P.R. Dutta, D.M. Cerasoli, and G. Kelsoe. 1998. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. V. Affinity maturation develops in two stages of clonal selection. *J Exp Med* 187:885-895.

Tanguay, D.A., T.P. Colarusso, S. Pavlovic, M. Irigoyen, R.G. Howard, J. Bartek, T.C. Chiles, and T.L. Rothstein. 1999. Early induction of cyclin D2 expression in phorbol ester-responsive B-1 lymphocytes. *J Exp Med* 189:1685-1690.

Taylor, A.L., S.L. Negus, M. Negus, E.M. Bolton, J.A. Bradley, and G.J. Pettigrew. 2007. Pathways of helper CD4 T cell allorecognition in generating alloantibody and CD8 T cell alloimmunity. *Transplantation* 83:931-937.

Taylor, D.O., L.B. Edwards, M.M. Boucek, E.P. Trulock, D.A. Waltz, B.M. Keck, and M.I. Hertz. 2006. Registry of the International Society for Heart and Lung Transplantation: twenty-third official adult heart transplantation report--2006. *J Heart Lung Transplant* 25:869-879.

Tiriveedhi, V., B. Gautam, N.J. Sarma, M. Askar, M. Budev, A. Aloush, R. Hachem, E. Trulock, B. Myers, A.G. Patterson, and T. Mohanakumar. 2013. Pre-transplant antibodies to Kalpha1 tubulin and collagen-V in lung transplantation: clinical correlations. *J Heart Lung Transplant* 32:807-814.

Tse, H.Y., S. Kanamori, W.D. Walsh, and T.H. Hansen. 1985. The murine bm12 gene conversion provides evidence that T cells recognize predominantly Ia conformation. *Proc Natl Acad Sci U S A* 82:7058-7062.

Turner, D.L., C.L. Gordon, and D.L. Farber. 2014. Tissue-resident T cells, in situ immunity and transplantation. *Immunol Rev* 258:150-166.

Tuzcu, E.M., S.R. Kapadia, R. Sachar, K.M. Ziada, T.D. Crowe, J. Feng, W.A. Magyar, R.E. Hobbs, R.C. Starling, J.B. Young, P. McCarthy, and S.E. Nissen. 2005. Intravascular ultrasound evidence of angiographically silent progression in coronary atherosclerosis predicts long-term morbidity and mortality after cardiac transplantation. *J Am Coll Cardiol* 45:1538-1542.

Uehara, S., C.M. Chase, W.H. Kitchens, H.S. Rose, R.B. Colvin, P.S. Russell, and J.C. Madsen. 2005. NK cells can trigger allograft vasculopathy: the role of hybrid resistance in solid organ allografts. *J Immunol* 175:3424-3430.

Valantine, H.A. 2003. Cardiac allograft vasculopathy: central role of endothelial injury leading to transplant "atheroma". *Transplantation* 76:891-899.

Valujskikh, A., E. Fedoseyeva, G. Benichou, and P.S. Heeger. 2002 Development of autoimmunity after skin graft rejection via an indirect alloresponse. *Transplantation* 73:1130-1137.

Valujskikh, A., Q. Zhang, and P.S. Heeger. 2006. CD8 T cells specific for a donor-derived, self-restricted transplant antigen are nonpathogenic bystanders after vascularized heart transplantation in mice. *J Immunol* 176:2190-2196.

van Besouw, N.M., C.R. Daane, L.M. Vaessen, B. Mochtar, A.H. Balk, and W. Weimar. 1997. Donor-specific cytokine production by graft-infiltrating lymphocytes induces and maintains graft vascular disease in human cardiac allografts. *Transplantation* 63:1313-1318.

van der Net, J.B., A. Bushell, K.J. Wood, and P.N. Harden. 2016. Regulatory T cells: first steps of clinical application in solid organ transplantation. *Transpl Int* 29:3-11.

Vantourout, P., and A. Hayday. 2013. Six-of-the-best: unique contributions of gammadelta T cells to immunology. *Nat Rev Immunol* 13:88-100.

Vassalli, G., A. Gallino, M. Weis, W. von Scheidt, L. Kappenberger, L.K. von Segesser, and J.J. Goy. 2003. Alloimmunity and nonimmunologic risk factors in cardiac allograft vasculopathy. *Eur Heart J* 24:1180-1188.

Victora, G.D., T.A. Schwickert, D.R. Fooksman, A.O. Kamphorst, M. Meyer-Hermann, M.L. Dustin, and M.C. Nussenzweig. 2010. Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. *Cell* 143:592-605.

Vincenti, F. 2016. Belatacept and Long-Term Outcomes in Kidney Transplantation. *N Engl J Med* 374:2600-2601.

Vinuesa, C.G., and J.G. Cyster. 2011. How T cells earn the follicular rite of passage. *Immunity* 35:671-680.

Vinuesa, C.G., M.A. Linterman, D. Yu, and I.C. MacLennan. 2016. Follicular Helper T Cells. *Annu Rev Immunol*

Vinuesa, C.G., I. Sanz, and M.C. Cook. 2009. Dysregulation of germinal centres in autoimmune disease. *Nat Rev Immunol* 9:845-857.

Vinuesa, C.G., S.G. Tangye, B. Moser, and C.R. Mackay. 2005. Follicular B helper T cells in antibody responses and autoimmunity. *Nat Rev Immunol* 5:853-865.

Vogelzang, A., H.M. McGuire, D. Yu, J. Sprent, C.R. Mackay, and C. King. 2008. A fundamental role for interleukin-21 in the generation of T follicular helper cells. *Immunity* 29:127-137.

von Boehmer, H., and F. Melchers. 2010. Checkpoints in lymphocyte development and autoimmune disease. *Nat Immunol* 11:14-20.

Vongwiwatana, A., A. Tasanarong, L.G. Hidalgo, and P.F. Halloran. 2003. The role of B cells and alloantibody in the host response to human organ allografts. *Immunol Rev* 196:197-218.

Wang, Y.H., and B. Diamond. 2008. B cell receptor revision diminishes the autoreactive B cell response after antigen activation in mice. *J Clin Invest* 118:2896-2907.

Wardemann, H., S. Yurasov, A. Schaefer, J.W. Young, E. Meffre, and M.C. Nussenzweig. 2003. Predominant autoantibody production by early human B cell precursors. *Science* 301:1374-1377.

Weis, M., T.N. Kledal, K.Y. Lin, S.N. Panchal, S.Z. Gao, H.A. Valantine, E.S. Mocarski, and J.P. Cooke. 2004. Cytomegalovirus infection impairs the nitric oxide synthase pathway: role of asymmetric dimethylarginine in transplant arteriosclerosis. *Circulation* 109:500-505.

Weis, M., and W. von Scheidt. 2000. Coronary artery disease in the transplanted heart. *Annu Rev Med* 51:81-100.

William, J., C. Euler, S. Christensen, and M.J. Shlomchik. 2002. Evolution of autoantibody responses via somatic hypermutation outside of germinal centers. *Science* 297:2066-2070.

William, J., C. Euler, E. Leadbetter, A. Marshak-Rothstein, and M.J. Shlomchik. 2005a. Visualizing the onset and evolution of an autoantibody response in systemic autoimmunity. *J Immunol* 174:6872-6878.

William, J., C. Euler, and M.J. Shlomchik. 2005b. Short-lived plasmablasts dominate the early spontaneous rheumatoid factor response: differentiation pathways, hypermutating cell types, and affinity maturation outside the germinal center. *J Immunol* 174:6879-6887.

Win, T.S., and G.J. Pettigrew. 2010. Humoral autoimmunity and transplant vasculopathy: when allo is not enough. *Transplantation* 90:113-120.

Win, T.S., S. Rehakova, M.C. Negus, K. Saeb-Parsy, M. Goddard, T.M. Conlon, E.M. Bolton, J.A. Bradley, and G.J. Pettigrew. 2009. Donor CD4 T cells contribute to cardiac allograft vasculopathy by providing help for autoantibody production. *Circ Heart Fail* 2:361-369.

Wu, L.C., D.S. Tuot, D.S. Lyons, K.C. Garcia, and M.M. Davis. 2002. Two-step binding mechanism for T-cell receptor recognition of peptide MHC. *Nature* 418:552-556.

Wucherpfennig, K.W., E. Gagnon, M.J. Call, E.S. Huseby, and M.E. Call. 2010. Structural biology of the T-cell receptor: insights into receptor assembly, ligand recognition, and initiation of signaling. *Cold Spring Harb Perspect Biol* 2:a005140.

Xu-Dubois, Y.C., J. Peltier, I. Brocheriou, C. Suberbielle-Boissel, A. Djamali, S. Reese, N. Mooney, Z. Keuylian, J. Lion, N. Ouali, P.P. Levy, C. Jouanneau, E. Rondeau, and A. Hertig. 2016. Markers of Endothelial-to-Mesenchymal Transition: Evidence for Antibody-Endothelium Interaction during Antibody-Mediated Rejection in Kidney Recipients. *J Am Soc Nephrol* 27:324-332.

Yang, Q., J. Jeremiah Bell, and A. Bhandoola. 2010. T-cell lineage determination. *Immunol Rev* 238:12-22.

Yen, M.H., G. Pilkington, R.C. Starling, N.B. Ratliff, P.M. McCarthy, J.B. Young, G.M. Chisolm, and M.S. Penn. 2002. Increased tissue factor expression predicts development of cardiac allograft vasculopathy. *Circulation* 106:1379-1383.

Yin, D., H. Zeng, L. Ma, J. Shen, H. Xu, G.W. Byrne, and A.S. Chong. 2004. Cutting Edge: NK cells mediate IgG1-dependent hyperacute rejection of xenografts. *J Immunol* 172:7235-7238.

Yoshida, S., A. Haque, T. Mizobuchi, T. Iwata, M. Chiyo, T.J. Webb, L.A. Baldridge, K.M. Heidler, O.W. Cummings, T. Fujisawa, J.S. Blum, D.D. Brand, and D.S. Wilkes. 2006. Anti-type V collagen lymphocytes that express IL-17 and IL-23 induce rejection pathology in fresh and well-healed lung transplants. *Am J Transplant* 6:724-735.

Yu, D., S. Rao, L.M. Tsai, S.K. Lee, Y. He, E.L. Sutcliffe, M. Srivastava, M. Linterman, L. Zheng, N. Simpson, J.I. Ellyard, I.A. Parish, C.S. Ma, Q.J. Li, C.R. Parish, C.R. Mackay, and C.G. Vinuesa. 2009. The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity* 31:457-468.

Yu, D., and C.G. Vinuesa. 2010. The elusive identity of T follicular helper cells. *Trends Immunol* 31:377-383.

Yu, G., X. Xu, M.D. Vu, E.D. Kilpatrick, and X.C. Li. 2006. NK cells promote transplant tolerance by killing donor antigen-presenting cells. *J Exp Med* 203:1851-1858.

Yu, W., H. Nagaoka, M. Jankovic, Z. Misulovin, H. Suh, A. Rolink, F. Melchers, E. Meffre, and M.C. Nussenzweig. 1999. Continued RAG expression in late stages of B cell development and no apparent re-induction after immunization. *Nature* 400:682-687.

Yuan, X., J. Paez-Cortez, I. Schmitt-Knosalla, F. D'Addio, B. Mfarrej, M. Donnarumma, A. Habicht, M.R. Clarkson, J. Iacomini, L.H. Glimcher, M.H. Sayegh, and M.J. Ansari. 2008. A novel role of CD4 Th17 cells in mediating cardiac allograft rejection and vasculopathy. *J Exp Med* 205:3133-3144.

Yung, S., and T.M. Chan. 2008. Anti-DNA antibodies in the pathogenesis of lupus nephritis--the emerging mechanisms. *Autoimmun Rev* 7:317-321.

Yurasov, S., H. Wardemann, J. Hammersen, M. Tsuiji, E. Meffre, V. Pascual, and M.C. Nussenzweig. 2005. Defective B cell tolerance checkpoints in systemic lupus erythematosus. *J Exp Med* 201:703-711.

Yusuf, I., R. Kageyama, L. Monticelli, R.J. Johnston, D. Ditoro, K. Hansen, B. Barnett, and S. Crotty. 2010. Germinal center T follicular helper cell IL-4 production is dependent on signaling lymphocytic activation molecule receptor (CD150). *J Immunol* 185:190-202.

Zecher, D., N. van Rooijen, D.M. Rothstein, W.D. Shlomchik, and F.G. Lakkis. 2009. An innate response to allogeneic nonself mediated by monocytes. *J Immunol* 183:7810-7816.

Zeisberg, E.M., O. Tarnavski, M. Zeisberg, A.L. Dorfman, J.R. McMullen, E. Gustafsson, A. Chandraker, X. Yuan, W.T. Pu, A.B. Roberts, E.G. Neilson, M.H. Sayegh, S. Izumo, and R. Kalluri. 2007. Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. *Nat Med* 13:952-961.

Zhang, Q., and E.F. Reed. 2016. The importance of non-HLA antibodies in transplantation. *Nat Rev Nephrol* 12:484-495.

Zhang, X., and E.F. Reed. 2009. Effect of antibodies on endothelium. *Am J Transplant* 9:2459-2465.

Zhao, J., and A.M. Lever. 2007. Lentivirus-mediated gene expression. *Methods Mol Biol* 366:343-355.

Zhao, J., G.J. Pettigrew, E.M. Bolton, C.R. Murfitt, A. Carmichael, J.A. Bradley, and A.M. Lever. 2005. Lentivirus-mediated gene transfer of viral interleukin-10 delays but does not prevent cardiac allograft rejection. *Gene Ther* 12:1509-1516.

Zhao, J., G.J. Pettigrew, J. Thomas, J.I. Vandenberg, L. Delriviere, E.M. Bolton, A. Carmichael, J.L. Martin, M.S. Marber, and A.M.L. Lever. 2002. Lentiviral vectors for delivery of genes into neonatal and adult ventricular cardiac myocytes <i>in vitro and <i>in vivo</i&gt. *Basic Research in Cardiology* 97:348-358.

Zhou, J., L. Qin, T. Yi, R. Ali, Q. Li, Y. Jiao, G. Li, Z. Tobiasova, Y. Huang, J. Zhang, J.J. Yun, M.M. Sadeghi, F.J. Giordano, J.S. Pober, and G. Tellides. 2015. Interferon-gamma-Mediated Allograft Rejection Exacerbates Cardiovascular Disease of Hyperlipidemic Murine Transplant Recipients. *Circ Res* 117:943-955.

Zhou, W., M.E. Medof, P.S. Heeger, and S. Sacks. 2007. Graft-derived complement as a mediator of transplant injury. *Curr Opin Immunol* 19:569-576.

Zotos, D., J.M. Coquet, Y. Zhang, A. Light, K. D'Costa, A. Kallies, L.M. Corcoran, D.I. Godfrey, K.M. Toellner, M.J. Smyth, S.L. Nutt, and D.M. Tarlinton. 2010. IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. *J Exp Med* 207:365-378.

Zou, Y., F.M. Heinemann, H. Grosse-Wilde, G. Sireci, Z. Wang, B. Lavingia, and P. Stastny. 2006. Detection of Anti-MICA Antibodies in Patients Awaiting Kidney Transplantation, during the Post-transplant Course, and in Eluates from Rejected Kidney Allografts by Luminex Flow Cytometry. *Human Immunology* 67:230-237.

Zou, Y., P. Stastny, C. Susal, B. Dohler, and G. Opelz. 2007. Antibodies against MICA antigens and kidney-transplant rejection. *N Engl J Med* 357:1293-1300.